UNIVERSITY OF ALBERTA

MOLECULAR MECHANISMS OF CYTOTOXIC T LYMPHOCYTE RECOGNITION AND ACTIVATION

BY

LIANJUN SHEN ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **MOLECULAR MECHANISMS OF CYTOTOXIC T LYMPHOCYTE RECOGNITION AND ACTIVATION** submitted by **LIANJUN SHEN** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **IMMUNOLOGY**.

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ABSTRACT

Cytotoxic T lymphocytes (CTL) are a subset of T cells typically expressing CD8 $\alpha\beta$ heterodimer glycoproteins that play a critical role in eliminating virus-infected cells and tumor cells. The CTL T cell receptor (TCR) specifically recognizes peptide antigen fragments displayed by the class I major histocompatibility complex (MHC) on the surface of target cells. CD8 can serve as a coreceptor in that it binds to the same MHC class I molecules as the TCR to facilitate efficient TCR signaling. In addition, CD8 can be activated by TCR stimulation to bind to class I molecules with high avidity, including class I not recognized by the TCR as antigenic complexes, to augment CTL responses and thus serve an accessory molecule function. Both CD8 functions are important for CTL activation and effector activities. The goals of this study are to understand the effects of MHC class I glycosylation patterns on CTL recognition, and to delineate the roles and mechanisms of MHC class I and CD8 interaction in CTL recognition and activation.

Two murine MHC class I K^b subsets differing significantly in their *N*-linked glycosylation have been separately isolated by immunoaffinity chromatography, and characterized by their differential reactivity to various anti-K^b antibodies. Both K^b subsets are effective ligands for CD8 accessory function, and both subsets contain appropriate peptides for alloreactive CTL recognition. However, only the normal glycosylated, but not the overglycosylated K^b subset, effectively triggers allo-CTL adhesion and responses. These results, for the first time, suggest that excessive glycosylation of MHC class I can affect TCR recognition, and provide insight into CTL surveillance of tumor cells.

The structure requirements for MHC class I to effectively serve as CD8 coreceptor and accessory ligands were investigated. The MHC class I molecules with a mutation in the α3 domain acid loop and their wild type counterparts were purified, immobilized on plastic and tested for their ability to trigger CTL adhesion and responses. It is found that the mutant class I molecules are effective ligands for TCR-triggered, activated CD8, indicating that the α3 acidic loop of class I is not essential for activated CD8 accessory molecule binding. However, the class I α3 mutation greatly affects CD8 coreceptor function as the mutant class I molecules are unable to initiate TCR-dependent signaling events to activate CD8. In addition, it is demonstrated that the CD8 β chain is not required for activated CD8 binding to MHC class I. A two-step model for CD8-MHC class I interaction during CD8-dependent CTL activation is proposed. The initial TCR/MHC/CD8 trimolecular complex formation, in which CD8 serves as a low avidity coreceptor, is essential for the subsequent expression of avidity-enhanced CD8 accessory function resulting in efficient CTL activation and effector function.

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ABBREVIATIONS

Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
ATCC	American Type Culture Collection
β_2m	β_2-microglobulin
BLT	N'-benzyloxycarbonyl-L-lysine thiobenzyl ester
BSA	Bovine serum albumin
Con A	Concanavalin A
cpm	Counts per minute
CPRG	Chlorophenol red β-galactoside
CTL	Cytotoxic T lymphocyte
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DOC	Deoxycholate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Endo F	Endoglycosidase F
Endo H	Endoglycosidase H
ER	Endoplasmic reticulum
E/T	Effector-to-target cell (ratio)
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
h	Hour
H-2	Mouse major histocompatibility complex
Hepes	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HLA	Human leukocyte antigen

HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
kD	Kilodalton
KIR	Killer-cell inhibitory receptor
LMP	Low-molecular mass polypeptide
mAb	Monoclonal antibody
2-ME	2-Mercaptoethanol
min	Minute
MHC	Major histocompatibility complex
M_r	Relative molecular mass
NANAse	Neuraminidase
NK	Natural killer (cell)
NP	Influenza nucleoprotein
NP-40	Nonidet-P40
OD	Optical density
OVA	Ovalbumin
PAGE	Polyacrylmide gel electrophoresis
PBS	Phosphate buffered saline
pD^b	D^b molecules purified from EL4 cells
pD^b/D^dLys	Purified chimeric D^b ($\alpha1\alpha2$) and D^d ($\alpha3$ with Glu227->Lys substitution) molecules
pD^b/D^dwt	Purified chimeric D^b ($\alpha1\alpha2$) and D^d ($\alpha3$ wild type) molecules
pK^b	K^b molecules purified from EL4 cells

$\text{pK}^b/\text{D}^d\text{Lys}$	Purified chimeric K^b ($\alpha 1\alpha 2$) and D^d ($\alpha 3$ with Glu227->Lys substitution) molecules
$\text{pK}^b/\text{D}^d\text{wt}$	Purified chimeric K^b ($\alpha 1\alpha 2$) and D^d ($\alpha 3$ wild type) molecules
PL	Poly-*N*-acetyllactosamine
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PTK	Protein tyrosine kinase
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SE	Serine esterase
SER	Serine esterase release
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TFA	Trifluoroacetic acid
Th	T helper (cell)
TNF	Tumor necrosis factor
U	Unit

CHAPTER I

INTRODUCTION

A. The Immune System: An Overview

Basic principles of immunity

The immune system is a highly evolved defense system of the vertebrates to protect them from invading pathogenic microorganisms and cancers. Two types of immunity are involved in the defense process. Natural immunity describes those defense mechanisms that are present prior to exposure to infectious microbes or other invaders, and are not enhanced by such exposures. The components include physiological barriers, phagocytes (macrophages, neutrophils), natural killer (NK) cells and some soluble factors. Acquired immunity is induced or stimulated by foreign or invading substances so the immune system is able to specifically recognize and selectively eliminate these invaders by mounting effective immune responses.

The hallmarks of all immune responses are specificity, diversity, memory and self-tolerance. Each lymphocyte is specific for only one antigen, and as a result, an individual has to express an extremely large number of antigen specificities, or lymphocyte repertoire, to cope with the antigenic universe. The immune system responds to the second or subsequent exposures to the same antigen more rapidly and more effectively. The immune system also acquires the ability to distinguish self from non-self, and is tolerant to self components. Together, these characteristics are fundamental for the immune system to respond to the tremendous complexity of invading pathogenic microorganisms rapidly, selectively and effectively without the destruction of self.

Acquired immunity is mediated by lymphocytes. Lymphocytes are blood cells that specifically recognize and respond to foreign antigens. All blood cells originate from a common self-renewing stem cell (Phillips, 1991) that becomes committed to differentiate into various lineages, i.e. erythroid, megakaryocytic, granulocytic, monocytic and lymphocytic. The acquired or specific immunity has two arms, humoral and cell-mediated immunity. Humoral immunity is mediated by antibodies or immunoglobulins (Ig) secreted by B lymphocytes or B cells. B cells mature in the bone marrow and leave the marrow expressing membrane-bound Ig (mIg). When specific mIg on the B cells encounters antigen, the B cells further differentiate into plasma cells which secrete large quantities of specific antibodies to carry out their effector functions. Antibody effector mechanisms include neutralization of toxins, activation of complement pathways, opsonization of pathogens and antibody-dependent cell mediated cytotoxicity.

While humoral immunity is effective in eliminating extracellular pathogens and their toxins, cell-mediated immunity specializes in the destruction of intracellular pathogens and cancer cells. T lymphocytes or T cells are responsible for this type of immune response. Unlike B cells, T cells do not recognize soluble antigens, but directly interact with cells bearing antigens displayed in the groove of major histocompatibility complex (MHC) molecules (see below). Upon recognition of specific antigen, some T cells directly kill the infected target cells, while other T cells produce soluble, low molecular weight proteins called cytokines, whose function is to promote the proliferation and differentiation of T cells as well as other cells, including B cells, macrophages and NK cells.

General features of T lymphocytes

1. Important T cell surface molecules

T cell receptor: The T cell receptor (TCR) is the structure on the T cell that specifically recognizes peptide fragments generated from foreign protein antigens displayed by self-MHC. This not only confers the specificity of T cell recognition, but also initiates the T cell activation process. The TCR is a covalently-linked heterodimer consisting of two polypeptide chains, the α and β chain. A small portion of T cell receptors are composed of γ and δ chains. The TCR α chain is a 40 to 60 kD glycoprotein and the β chain is a 40 to 50 kD glycoprotein, and both have very short cytoplasmic tails. They are members of the Ig superfamily and organized with a membrane-distal variable (V) and a membrane-proximal constant (C) region (Davis and Bjorkman, 1987). The Ig superfamily includes proteins that contain domain(s) homologous to the Ig V or C domains. Developmentally, the TCR undergoes germ-line DNA rearrangement of V(D)J segments to generate TCR diversity similar to immunoglobulins (Davis and Bjorkman, 1987).

TCR-associated invariant chain complex: The TCR αβ heterodimer is non-covalently associated with a complex of several invariant chains. This complex consists of CD3 (which includes γ, δ and ε chains), as well as ζ chain and η chain (Weiss and Littman, 1994). A TCR receptor complex is composed of a TCR αβ heterodimer, a γε heterodimer, an εδ heterodimer and a disulfide-linked ζζ homodimer or a ζη heterodimer. In addition to facilitating the assembly and surface expression of TCR αβ heterodimer, these invariant chains are indispensable for T cell activation by transducing TCR-initiated signals to the inside of the cell, as the TCR αβ heterodimer is unable to signal by itself.

Accessory molecules: T cells also express membrane molecules which are not involved in antigen recognition but play an important role in T cell activation (Springer, 1990; van Seventer et al., 1991, Smith et al., 1994). These molecules are collectively called accessory molecules. By binding to their respective ligands on antigen presenting cells (APC) or target cells, accessory molecules can function as adhesion molecules which strengthen the interaction between T cells and APC or target cells. Many accessory molecules can also provide costimulatory signals by functioning as signaling molecules to amplify TCR signals and/or to transduce distinct signals for T cell activation. The list of the accessory molecules is large and expanding, but they generally fall into three categories: the integrin family, the Ig superfamily and the TNF/TNF receptor superfamily (Springer, 1990; van Seventer et al., 1991, Smith et al., 1994). Several accessory molecule/ligand pairs have been shown to participate in T cell-APC or target interactions: lymphocyte function-associated antigen (LFA)-1 and intercellular adhesion molecule (ICAM) 1, 2, 3; CD2 and LFA-3; very late antigen (VLA) and extracellular matrix; CD28/CTLA-4 and the B7 family (Springer, 1990; van Seventer et al., 1991; Mondino and Jenkins, 1994). Among these molecules, LFA-1 and VLA are members of the integrin family; while CD28/B7, CD2/LFA-3 and ICAMs belong to the Ig superfamily. The emerging TNF/TNF receptor gene family members expressed on T cells, including CD40 ligand (Van Kooten and Banchereau, 1996), CD30 (Del Prete et al., 1995), CD27 (Hintzen et al., 1994), and 4-1BB (DeBenedette et al., 1995), have also been shown to participate in T cell differentiation, proliferation and activation. Two special molecules CD4 and CD8 are described below.

CD4 and CD8 coreceptor/accessory molecules: Both CD4 and CD8 contain Ig-like extracellular domains and thus belong to the Ig superfamily.

CD4 is a monomeric 55 kD transmembrane glycoprotein. CD8 consists of a disulfide-linked αβ heterodimer or as an αα homodimer. The α chain is a 38 kD glycoprotein and the β chain is a 30 kD glycoprotein (Leahy, 1995). Ligands for CD4 and CD8 are the MHC class II and class I molecules, respectively. The feature that makes them unique from the rest of the accessory molecules is that they both serve as coreceptors. Coreceptor denotes that the CD4 or CD8 molecule interacts with the same MHC molecule that the TCR interacts with to facilitate efficient TCR signaling (Janeway, 1992). In addition to serving as a coreceptor, both CD4 and CD8 can function as accessory molecules by interacting with their respective ligands, MHC class II and class I molecules that are not engaged by TCR (Gay et al., 1987; O'Rourke et al., 1990; Takeshita et al., 1993; Huang et al., 1997). Both the coreceptor and accessory functions of CD4 and CD8 contribute to the recognition and activation process of $CD4^+$ and $CD8^+$ T cell subsets, respectively.

2. T cell subsets

CD4 and CD8 are expressed on mature T cells in a mutually exclusive fashion and correlate with T cell MHC-restriction patterns (Swain et al., 1983). $CD4^+$ T cells recognize antigen in association with MHC class II molecules, whereas $CD8^+$ T cells recognize antigen presented by MHC class I molecules. T cells expressing CD4 are mostly T helper (Th) cells, which produce cytokines as regulators of immune responses. Th cells can be further divided into Th1 and Th2 cells based on their cytokine production patterns (Mosmann and Coffman, 1989; Abbas et al., 1996). The Th1 cells predominantly produce interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-β, and are responsible for classical cell-mediated functions. The Th2 cells typically produce IL-4, IL-5, IL-10 and IL-13, and are more effective as helpers for B cell activation. $CD8^+$ T cells are generally cytolytic, and are called cytotoxic T lymphocytes (CTL). Similar to Th cells, $CD8^+$ T cells can also be induced to develop into two subsets which secrete Th1- or Th2-like cytokines (Sad et al., 1995). CTL play a major role in eliminating virally-infected cells or tumor cells and will be discussed in detail below.

T cell maturation in the thymus

T cells originate from the bone marrow stem cells, and their progenitors migrate to the thymus where they mature. In the thymus, the immature T cells, or thymocytes, proliferate and differentiate along different developmental pathways that can be identified by distinctive patterns of cell surface marker expression. It is during thymocyte development that the T cells undergo the gene rearrangements that produce the TCR, and the positive and negative selection that shapes the mature T cell repertoire (von Boehmer et al., 1989).

It is generally accepted that there are several stages of T cell development in the thymus as defined by the surface expression of TCR/CD3, CD4 and CD8 molecules (von Boehmer, 1990). The earliest fetal thymocytes do not express TCR/CD3, CD4 and CD8 (double negative, DN). A very small portion of these DN cells make successful rearrangements of both the γ and δ genes and differentiate into $TCR/CD3^+$ $\gamma\delta$ T cells (Allison and Havran, 1991), while the vast majority of the DN cells undergo TCR β and α chain gene rearrangements and develop into $\alpha\beta$ TCR^+ T cells. The productive rearrangements of TCR β and α chain genes plus the expression of both CD4 and CD8 lead to the appearance of $TCR/CD3^+$, $CD4^+CD8^+$ (double positive, DP) thymocytes. The majority of the thymocytes are of this DP phenotype. It is believed that this is the stage that thymic selection occurs (von Boehmer, 1990). Negative selection eliminates thymocytes that have high affinity for self-MHC alone or with self-antigens, and ensures that mature T cells are self-tolerant. Most DP thymocytes are thought to be negatively selected and to undergo death by apoptosis or programmed cell death, a phenomenon characterized by DNA fragmentation (Cohen, 1993). It is believed that thymocyte receptors interact with MHC class I or class II molecules expressed on the bone marrow-derived cells in the thymus, and those thymocytes with high affinity for self-MHC are eliminated (Anderson et al., 1996). Positive selection permits the maturation and expansion of thymocytes whose receptors are capable of binding with antigen plus self-MHC with moderate affinity, and mature CD4 or CD8 single positive T cells leave the thymus for the periphery. The result of positive selection is MHC-restriction, in that T cells recognize antigen complexed with self-MHC molecules. Thymic epithelial cells appear to be involved in the positive selection of thymocytes (Anderson et al., 1996).

CD4 and CD8 molecules influence the T cell maturation and positive selection processes, because both CD4 and CD8 function as coreceptors with the TCR for their respective ligand MHC class II and class I, augmenting TCR engagement and/or signaling (Yeung et al., 1994). As a result of thymic maturation, $CD4^+$ T cells recognize antigen displayed by MHC class II molecules, while the $CD8^+$ T cells recognize antigen presented by MHC class I molecules.

B. The Major Histocompatibility Complex

The major histocompatibility complex (MHC) is a large genetic region encoding the class I and class II MHC molecules as well as other proteins including LMP (low-molecular mass polypeptide) and TAP (transporter associated with antigen presentation) which are involved in antigen processing and presentation by MHC molecules. In humans, MHC is referred to as the human leukocyte antigen (HLA) and located on chromosome 6; while in mice, it is referred to as H-2 and located on chromosome 17. The classical class I molecules are encoded by the K, D and L loci in mice and by A, B, and C loci in humans. The class II MHC molecules are encoded by the I-A and I-E loci in mice and D loci in humans. Each locus has many different allelic forms, and the combination of alleles present on one chromosome is termed the MHC haplotype.

The MHC class I molecules are expressed on the surface of nearly all nucleated cells. Class I molecules are expressed on the cell surface as heterodimers consisting of two subunits, an MHC-encoded 44 to 47 kD heavy chain and a non-MHC-encoded 12 kD light chain, β2-microglobulin (β2m). The heavy chain is a transmembrane glycoprotein consisting of three external domains (α1, α2 and α3), a transmembrane domain and a cytoplamic tail (Robinson and Kindt, 1989). There are one to three *N*-linked glycosylation sites in class I heavy chains. The α1 and α2 domains of the class I molecules are highly polymorphic, while the α3 domain is conserved. The β2m is non-covalently associated with the α3 domain of the heavy chain and is required for the expression of class I complexes on the cell surface. The crystal structure of MHC class I reveals that the α1 and α2 domains form a platform of β strands supporting two parallel strands of α-helices (Bjorkman et al., 1987). This structure forms a groove on the top surface of the class I molecule and is the peptide binding cleft (Bjorkman et al., 1987). The cleft contains "pockets" which accommodate high affinity peptide binding (Madden, 1995). Recent crystallographic analyses suggest that the TCR contacts both α-helices of MHC class I and also the bound peptide itself

(Garboczi et al., 1996; Garcia et al., 1996a). The membrane-proximal α3 domain posseses a critical binding site for the CD8 coreceptor (Salter et al., 1990).

The MHC class II molecules are primarily expressed on the surface of antigen presenting cells, namely macrophages, dendritic cells, and B cells. Class II molecules are noncovalently-linked heterodimeric transmembrane glycoproteins consisting of an α chain (25 to 33 kD) and a β chain (24 to 29 kD). Class II α and β chains are structurally similar and each contains two external domains: α1 and α2, and β1 and β2. The polymorphic residues are clustered in the membrane-distal domain (α1 and β1) of both chains, and the membrane-proximal α2 and β2 domains are highly conserved. Mutational analyses have shown that the β2 domain is a binding site for the CD4 coreceptor protein (Cammarota et al., 1992; Konig et al., 1992), and recent data indicate that the α2 domain is also involved in CD4 interaction (Konig et al., 1995). The overall three-dimensional structure of MHC class II molecules is very similar to that of class I (Brown et al., 1993). A potential difference is that class II molecules can form dimers of the αβ heterodimers, however, the biological relevance of this "dimer of dimers" is still not fully elucidated (Ploegh and Benaroch, 1993). A recent report suggest that murine class II dimers of heterodimers can be expressed on the cell surface and are more efficient in stimulating low affinity T cell responses (Schafer and Pierce, 1994)

Some peptides bound by MHC class I and class II have been characterized. For both MHC types, peptides are held in a largely extended conformation and run the length of the cleft. The MHC class I molecules typically associate with a short peptide of 8 to 10 residues (Rotzschke and Falk, 1989; Rammensee et al., 1993), in which two to three residues are conserved and bind into the deep pockets of the class I cleft (Madden, 1995).

The class II molecules bind longer peptides which are 12 to 24 residues (Rudensky et al., 1991). This difference in bound peptide length could be partly due to the class I peptide binding groove being "closed" or occluded at both ends by heavy chain amino acid residues, while the class II molecule cleft is unobstructed or "open" on both ends. Differences in bound peptide length is also because the generation of antigenic peptides is different for the two classes of MHC (Brodsky and Guagliardi, 1991; Germain and Margulies, 1993).

The MHC genes are highly polymorphic as there are multiple alleles at a given MHC locus in a species. As a result, the encoded MHC proteins are highly polymorphic as well. The polymorphic residues do not distribute randomly along the entire MHC peptide chain, but instead cluster inside or near the peptide binding groove in both class I and class II MHC molecules, influencing the selection of peptides that bind. The substantial degree of MHC polymorphism results in tremendous diversity of MHC molecules and the repertoire of bound peptides within a species. This allows the immune system to be able to detect and eliminate pathogenic microorganisms that are constantly mutating (McFadden and Kane, 1994; Parham and Ohta, 1996).

C. Antigen Processing and Presentation to T Lymphocytes

T cells recognize peptides derived from foreign antigens displayed within the peptide binding grooves of the MHC molecules. Earlier studies showed that the MHC class I and class II molecules are distinct in their requirements for antigen sources, sensitivity to various inhibitors, and the T cell subsets to which they present antigen (Braciale et al., 1987; Peters et al., 1991; Brodsky and Guagliardi, 1991; Germain and Margulies, 1993).

suggesting that different antigen processing and presentation pathways are involved. It is now generally accepted that class I MHC molecules bind peptides generated from endogenous antigens and present them to $CD8^+$ CTL, whereas class II MHC molecules bind peptides derived from exogenous antigens and present them to $CD4^+$ T cells. Antigen processing and presentation pathways must include entry of proteins into the pathway, degradation of proteins, assembly of peptide-MHC complexes and transport of the assembled complexes to the cell surface.

Classical class I pathway

The endogenous protein antigens targeted for presentation by MHC class I molecules include normal cellular proteins, viral proteins and intracellular bacterial proteins (Heemels and Ploegh, 1995; York and Rock, 1996). The majority of presented peptides are generated by degradation of proteins in the cytosol by a large multicatalytic proteolytic proteasome complex (Goldberg and Rock, 1992; Rock et al., 1994). The proteasome can contain two MHC region-encoded subunits, LMP2 and LMP7 (low-molecular mass polypeptide) (Brown et al., 1991). The expression of these two subunits of the proteasome are inducible by IFN-γ and they can alter the catalytic activities of proteasome complexes and enhance the presentation of at least some antigens by increasing the yield of specific peptides through altered proteasome processing (Fehling et al., 1994; Van Kaer et al., 1994). The resulting peptides in the cytosol are transported across the endoplasmic reticulum (ER) membrane in an ATP-dependent manner by a heterodimeric protein designated TAP (transporter associated with antigen presentation), (Monaco, 1992). TAP is a member of the ATP-binding cassette family of transport proteins and is encoded by genes in the MHC region.

The class I heavy chain and β2m are cotranslationally translocated into the ER where they assemble, and are loaded with antigenic peptides. This process involves a series of MHC class I interactions with ER chaperone proteins and possibly other proteins (Williams and Watts, 1995; Solheim et al., 1997). The newly synthesized unfolded class I heavy chain initially interacts with an ER resident lectin-like chaperone protein calnexin and then associates with β2m (Williams and Watts, 1995). In the human system, the class I heavy chain-β2m complex subsequently interacts with another calnexin-related protein calreticulin and further associates with TAP in the ER membrane (Sadasivan et al., 1996). The association between calreticulin-class I complex with TAP is bridged by a newly identified glycoprotein termed tapasin (Sadasivan et al., 1996). In the mouse system, however, the sequential interaction of class I with calreticulin, tapasin and TAP has not been directly demonstrated. Instead, it is found that class I heterodimer form complex with calnexin and TAP before peptide loading (Suh et al., 1996). In both human and mouse systems, the TAP-associated class I complexes are retained in the ER and loaded with peptides. Once the MHC class I/β2m/peptide trimolecular complexes are formed, they are dissociated from TAP, released from the ER and transported through the Golgi apparatus to the cell surface for CTL surveillance (York and Rock, 1996).

Class II pathway

Antigen presenting cells, such as macrophages, dendritic cells and B cells can internalize exogenous antigens by phagocytosis, or endocytosis, or both. These antigens enter the endocytic pathway where they are degraded into oligopeptides of 12 to 24 residues by abundant proteases including various cathepsins resident in the endosomal/lysosomal compartments

(Cresswell, 1994). MHC class II molecules are synthesized and co-translationally translocated to the ER where they form $\alpha\beta$ heterodimers and associate with a third chain, termed the invariant chain (Ii chain) (Neefjes and Ploegh, 1992). The association between the $\alpha\beta$ heterodimer and the Ii chain prevents peptides in the ER from binding to class II peptide binding cleft (Teyton et al., 1990). The targeting sequence in the N-terminal cytoplasmic portion of the type II transmembrane protein Ii chain directs the α-β-Ii complex to the endocytic pathway (Lotteau et al., 1990; Teyton and Peterson, 1992). The location and mechanism of peptide loading into class II molecules is not clear, but it is suggested peptide loading occurs in an early lysosomal compartment termed MIIC (MHC class II compartment) (Harding, 1996). In this specialized compartment, the Ii chain is cleaved leaving a fragment of the Ii chain termed CLIP (class II-associated invariant chain peptide) still occupying the peptide binding cleft until processed antigenic peptide binds to the class II molecule (Harding, 1996). After peptide loading, class II molecules are transported to the cell surface for recognition by $CD4^+$ T cells.

D. CTL-Mediated Cytotoxicity

CTL recognize peptide antigens presented by class I MHC molecules, and usually express CD8 as a result of the thymic selection process. CTL have an important role in recognizing and eliminating altered-self cells, including virus-infected cells, malignant cells, and allogeneic cells. There are two phases of a CTL-mediated response: first is the activation of a precursor CTL into an effector CTL; the second is the destruction of the target cells by effector CTL.

Generation of effector CTL

After T cells have completed their development in the thymus, they migrate into the bloodstream and circulate until they encounter antigen. Naive CTL do not contain granules and are incapable of lysing target cells; they must be stimulated or primed to proliferate and differentiate into functional effector CTL. The effective priming of CTL precursors generally requires two signals (Schwartz, 1990). One signal is generated by specific TCR recognition of peptide presented by MHC class I on the surface of an APC or target cell. However, this signal is usually not sufficient to induce CTL precursors to develop into effector CTL, but rather results in partial activation and a state of anergy. The second signal is primarily provided by CD28-B7 interaction. CTL express the important costimulatory molecule, CD28, which interacts with members of the B7 family expressed on APC or target cells. The interaction between CD28 and B7, in combination with the TCR/MHC interaction, can activate CTL precursors to produce IL-2, and IL-2 facilitate CTL differentiation in an autocrine fashion (Linsley and Ledbetter, 1993). Cytokines, primarily IL-2, provided by CD4$^+$ T cells in a paracrine fashion can also be important for the differentiation of a CTL precursor into an effector CTL. At the end of this activation process, the agranular CTL precursors differentiate into functional effector CTL which contain granules.

Molecular mechanisms of CTL killing

When an effector CTL encounters a target cell that displays the peptide-MHC complex for which its TCR is specific, the CTL killing machinery is activated and the target cell is lysed. At the initial stage, CTL randomly contact target cells, and the binding is non-antigen-specific and of low avidity. This initial CTL-target conjugate formation is mediated by several accessory molecule/ligand pairs including LFA-1/ICAMs and CD2/LFA-3. Once the TCR on the CTL recognizes the specific peptide-MHC class I complex, the TCR transduces a signal to upregulate the affinity of several accessory molecules including LFA-1 and CD8. This allows CTL to form a higher avidity conjugate with the appropriate target cell and deliver the "lethal hit". The CTL then dissociates from the target cell and binds another target cell. The target cells productively engaged by CTL undergo death by apoptosis or programmed cell death which is characterized by unique morphological changes and DNA fragmentation (Cohen, 1993; Schwartz and Osborne, 1993). Two major pathways have been implicated in CTL-mediated cytolysis.

1. Granule exocytosis model

Following antigen-specific CTL-target conjugate formation, effector CTL undergo reorientation of cytoplasmic granules. The granules are concentrated at the junction of CTL and target cell, and the contents released by exocytosis (Henkart et al., 1993). Perforin and granzymes contained in the CTL granules can be critical for CTL cytotoxicity. Perforin is a 65-75 kD polypeptide that, in a calcium-dependent manner, polymerizes to form large cylindrical structures which insert into the lipid bilayer of the target. *In vitro* experiments demonstrated that the purified perforin can form "holes" in target cell plasma membranes. However, this does not explain the fact that target cells die by apoptosis. A family of granule-localized serine proteases/esterases designated as granzymes has been shown to be able to initiate target cell DNA fragmentation (Shi et al., 1992). Together, these results indicate that the entry of granzymes into target cells, facilitated by the pore-forming protein, perforin, is a likely mechanism of CTL cytotoxicity.

2. Fas-mediated cytotoxicity

Recently, a calcium-independent, Fas-mediated mechanism of CTL cytotoxicity has been identified. Fas (or APO-1, CD95) is a transmembrane protein of the TNF receptor gene family (Atkinson and Bleackley, 1995). It is expressed on a wide variety of cell types including T cells. Crosslinking of Fas results in apoptotic death of Fas$^+$ targets. The finding that Fas ligand, a 31 kD type II membrane protein (a member of the TNF family), is expressed on the CTL cell surface lends strong support for the involvement of Fas in CTL-mediated cytotoxicity (Suda et al., 1993 & 1995). Transfection of Fas ligand into non-cytolytic cells renders these cells a potent cytotoxic activity towards Fas$^+$ target cells (Suda et al., 1993). Upon CTL TCR engagement of specific peptide/MHC complexes, Fas ligand expression on CTL is upregulated (Suda et al., 1995; Glass et al., 1996). Fas ligand interaction with Fas on the target cells triggers a death signal resulting in DNA fragmentation and death of the targets (Henkart, 1994).

However, the relative contribution of granule exocytosis and Fas-mediated cytotoxicity in host defense is unclear. Although both mechanisms are functional in *in vitro* assays, it is suggested that the Fas-mechanism may be more involved in the regulation of immune responses and less in host defense against pathogens (Kagi et al., 1996).

Allogeneic recognition by CTL

T cells of the immune system mount a vigorous rejection response towards allogeneic grafts, i.e. the tissues or cells of a different member of the same species. It is clear that the antigens (alloantigens) that induce this strong response are the foreign MHC molecules. Alloreactive T cells can be generated both *in vitro* and *in vivo* by stimulating responder cells with MHCdisparate stimulator cells. The molecular mechanism of allorecognition has been a puzzle, since it does not obey the rule of MHC restriction. Furthermore, it has been estimated that a very high percentage (about 2 -10%) of all T cells are alloreactive relative to the number of responding T cells to a nominal antigen, 1 in 10^4-10^6. It is now believed that allo-MHC recognition is a cross-reaction of a normal CTL TCR that was selected to recognize self MHC plus foreign peptide. CTL allorecognition is largely peptide-dependent (Sherman and Chattopadhyay, 1993). An allo-specific CD8$^+$ CTL clone usually recognizes one or a small number of specific endogenously processed peptide(s) complexed with allo-class I molecules (Rotzschke et al, 1991). Collectively, a population of allo-reactive CTL respond to allo-class I and a diverse array of endogenous bound peptides. This may explain why such a high percentage of T cells are alloreactive.

E. Natural Killer Cells

NK cells are distinct cytolytic lymphocytes and play an important role in eliminating virus-infected cells and tumor cells. They constitute less than 5% of the circulating lymphocyte population. NK cells contain granules in their cytosol and lyse target cells by exocytosis. NK cells are distinct from CTL in several ways. Phenotypically, NK cells do not express TCR/CD3 complexes, or CD8, but express CD16 (FcRγIII). Functionally, they do not require prior antigen priming for cytolytic activity, and lyse targets in an MHC-unrestricted manner. NK cells can also lyse target cells by ADCC (antibody-dependent cell-mediated cytotoxicity) through their surface FcRγIII. It is now generally accepted that NK cells do not express a specific "NK antigen receptor" analogous to the TCR, but instead express a number of cell surface receptors that interact with their respective ligands on the target cells and regulate NK cell activity (Gumperz and Parham, 1995).

NK receptors encoded by the NK gene complex

Recently, studies have been focused on the NK gene complex (NKC) which is found on chromosome 12 in humans and chromosome 6 in mice (Yokoyama and Seaman, 1993). NKC encodes a number of proteins that are implicated in NK cell target recognition and function. NKC products include Ly-49 and NKR-P1 multigene families in mice, and NKR-P1 and CD94 in humans. Rat NKR-P1 has been shown to bind carbohydrate structures on target cells and to activate NK cells. Human CD94 is a member of the C-type lectin family, and inhibits NK function upon interaction with its ligand, HLA class I (Gumperz and Parham, 1995).

The mouse Ly-49 family has at least 9 members, Ly-49 A to I. Ly-49 are type II integral membrane proteins homologous to the C-type lectin family (Yokoyama and Seaman, 1993; Gumperz and Parham, 1995). They are expressed on the NK cell surface as disulfide-linked dimers. Each subunit of an Ly-49 dimer has an extracellular carbohydrate recognition domain (CRD), an external stalk, a transmembrane domain and a cytoplasmic tail. The CRD are potential carbohydrate binding sites. The ligands for Ly-49 are the H-2 class I molecules, and each Ly-49 member may be specific for a different spectrum of H-2 class I allele products (Lanier and Phillips, 1996; Colonna, 1996). For instance, Ly-49A is specific for H-2 D^d and D^k, and Ly-49C is specific for H-2 K^b and H-2^d. Ly-49 molecules interact with H-2 class I molecules and generate an inhibitory or negative signal through their cytoplasmic tails, which results in the inhibition of Ly-49-expressing NK killing. The intracytoplasmic tail has a consensus sequence designated immunoreceptor tyrosine-based inhibitory motif (ITIM) for most members of the Ly-49 family. It has been shown that the Ly-49 intracellular domain associates with a cytoplasmic tyrosine phosphatase SHP-1 via its ITIM motif, and inhibits the tyrosine phosphorylation events normally associated with NK cell activation (Nakamura et al., 1997; Renard et al., 1997).

Killer cell inhibitory receptors

In humans, the NK cells express a family of closely related molecules that generally inhibit human NK cell lysis of targets, hence they are designated killer cell inhibitory receptors (KIR) (Lanier and Phillips, 1996). Unlike the Ly-49 C-type lectin family in mice, KIRs are single chain Ig-superfamily proteins with 2 to 3 Ig domains. One of the KIR members, p58, recognizes HLA class I and discriminates between two HLA-C allotypes via its first Ig domain (Winter and Long, 1997). It has been reported that KIR specifically recognizes polymorphic sites in the HLA class I $\alpha 1$ domain (Gumperz et al., 1995). KIR also mediates the inhibitory signal(s) via its cytoplasmic ITIM by associating with tyrosine phosphatase SHP-1 (Binstadt et al., 1996; Renard et al., 1997).

F. MHC Class I Molecules as CTL and NK Recognition Structures

MHC class I molecules are ligands for CTL and NK cell receptors

MHC class I molecules are expressed on the surface of most nucleated cells and they participate in immune recognition and responses in several ways. MHC class I molecules serve as CTL TCR ligands by presenting specific peptides for CTL recognition. During CTL activation, class I molecules also function as ligands for CD8 coreceptor/accessory molecules to facilitate CTL response and will be discussed later. In addition to a role in

CTL recognition and activation, MHC class I also interact with a number of NK receptors to regulate NK cell activity. MHC class I molecules are the recognition structures of the murine Ly-49 family and human KIR which mediate NK inhibition. The roles and ligand interactions of MHC class I molecules in immune recognition/regulation are summarized in the following table and described below.

	MHC class I ligands			
MHC class I	TCR	CD8	Ly-49	KIR
Contact sites	α1α2	α3	α1α2	α1
Peptide requirement	Yes	No	Yes	Yes
Peptide specificity	Yes	No	No	Yes
CHO* involvement	?	No	Yes?	No
Function	TCR activation	Coreceptor/ Accessory	NK inhibition	NK inhibition

(*CHO: carbohydrate)

MHC class I recognition by TCR

There are three hypervariable regions or CDR (complementarity determining regions) in the V regions or V(D)J junctions in both TCR α and β chains and they are identified to be the sites for binding of MHC-peptide complex. Based on the information provided by the crystal structures of MHC class I molecules, mutational analysis of class I has shown that the TCR contacts a limited region of class I on both helices formed by the α1 and α2 domains (Sun et al., 1995; Smith and Lutz, 1997). Recent crystal structures of TCR complexes with MHC/peptide have provided clearer pictures of how the TCR interacts with MHC class I (Garcia et al., 1996a; Garboczi et al., 1996). The TCR contacts peptide-MHC class I complex diagonally, which probably facilitates an interlocking of TCR and MHC molecules. The TCR Vα and Vβ

CDR cover most part of the class I peptide binding site with contacts to both the α helices and the bound peptide, with TCR Vα spanning the left end of the peptide binding groove and TCR Vβ covering the right half (Parham, 1996b). The structural information not only has direct implications for understanding MHC class I interaction with TCR, but also sheds light into how MHC class I can potentially interact with other ligands.

MHC class I regulation of NK function

It has long been observed that NK function is inversely correlated with MHC class I expression on the target cells (Trinchieri, 1994). Generally, NK cells lyse targets which have no or low MHC class I expression. This has led to the "missing self" hypothesis. The finding that MHC class I molecules are ligands for the inhibitory NK receptors, murine Ly-49 and human KIR, provides a biochemical basis for these observations. Currently, it is not completely understood regarding the structural basis for MHC class I molecule interaction with NK receptors, and the role for MHC class I bound peptides as well as MHC class I glycosylation in NK receptor recognition.

In contrast to TCR/MHC class I interaction, little is known about how Ly-49 and KIR interact with MHC class I molecules. This is largely due to a lack of structural information about these NK receptor molecules, and only limited mutational analysis performed. Based on antibody blocking experiments, it is found that Ly-49 molecules interact with the α1α2 domains of H-2 class I molecules (Kane, 1994; Brennan et al., 1996). By comparing KIR interactions with various HLA-B allele products which differ in their α1 domain sequences, it has been shown that human KIR specifically recognizes amino acid residues in the α1 domain of HLA class I (Gumperz et al., 1995). Since both KIR and Ly-49 recognize the MHC class I α1α2 domains which form the peptide binding groove, it is likely the peptide contents may affect KIR and Ly-49 recognition of MHC class I (Karre, 1995). In the murine system, Ly-49A does not recognize peptide-empty D^d, and peptides are needed to stabilize the class I complexes for Ly-49A recognition. However, peptide specificity has not been demonstrated (Correa and Raulet, 1995; Orihuela et al., 1996). Hence it is suggested that Ly-49 recognizes a peptide-induced conformational determinant of H-2 class I complex (Orihuela et al., 1996). In contrast, in the human system, KIR recognizes and binds HLA class I only when class I molecules are bound by certain peptides (Malnati, et al., 1995; Rajagopalan and Long, 1997), suggesting that KIR recognition of MHC class I is peptide-selective. The potential role of glycosylation of MHC class I on KIR and Ly-49 recognition is discussed below.

G. Glycosylation of MHC Class I and Its Role in Immune Recognition

N-*linked glycosylation of MHC class I*

MHC class I molecule heavy chains are *N*-linked glycosylated. All human HLA-A, B, and C have only one *N*-linked glycosylation site, at Asn86, in the $\alpha1$ domain (Barber et al., 1996). Murine MHC class I molecules usually have 2 or 3 *N*-linked glycans (Watts et al., 1989; Robinson and Kindt, 1989). For instance, H-2 K^b, K^k and D^d have two *N*-linked glycosylation sites at Asn86 ($\alpha1$ domain) and Asn176 ($\alpha2$ domain), while K^d, D^b, D^k and L^d have an additional third site at Asn256 located in the $\alpha3$ domain.

It has long been observed that the same MHC class I allele products expressed at the cell surface apparently display different sizes and charges as shown on two-dimensional PAGE analysis (Jones, 1977; Gilmer, 1982; Le and Doyle, 1982; Swiedler et al., 1985). Later it was demonstrated that this "microheterogeneity" of MHC class I is due to heterogeneous *N*-linked carbohydrate addition to the class I polypeptide backbones (Jones, 1977; Gilmer, 1982; Le and Doyle, 1982; Swiedler et al., 1985). However, there is limited information regarding the composition, structure and sequence of the carbohydrate side chains. The *N*-linked oligosaccharides attached to the surface-expressed MHC class I molecules are of the mature complex type, consisting of mannose, *N*-acetylglucosamine, galactose and sialic acid (Swiedler et al., 1985; Hayes et al., 1995; Barber et al., 1996). Typically, each *N*-glycan has 12 to 15 monosaccharide residues. The predominant branching pattern of the oligosaccharides is biantennary, however, tri- and tetra-antennary structures are also observed (Swiedler et al., 1985; Hayes et al., 1995; Barber et al., 1996).

Role of MHC class I glycosylation on CTL recognition

N-linked glycosylation plays an important role in MHC class I biosynthesis, as elimination of *N*-linked glycosylation sites by site-directed mutagenesis results in a greatly reduced level of MHC class I expression at the cell surface (Shiroishi et al., 1985; Miyasaki et al., 1986; Barbosa et al., 1987). However, the exact role(s) of MHC class I glycosylation on CTL recognition and activation is not well understood. Goldstein and Mescher (1985) have shown that enzymatic removal of *N*-linked glycosylation on purified murine class I K^k molecules did not affect its ability to induce allo-CTL response. Abrogation of *N*-linked glycosylation acceptor sites on L^d by site-directed mutagenesis (Shiroshi et al., 1985; Miyazaki et al, 1986) altered L^d recognition by several specific monoclonal antibodies (mAb), but did not affect the allo-CTL lysis of the transfectants expressing these mutant molecules. Similar results were observed for human HLA-B7 (Barbosa et al., 1987). These earlier studies indicate that the oligosaccharide structures on MHC class I are not required for allo-CTL recognition. However, it is unclear what influence the complexity of *N*-linked glycosylation on MHC class I may have on CTL recognition and effector functions (Powell et al., 1985; Degen et al., 1989).

Role of N-*linked glycosylation on NK cell recognition*

In addition to serving as an antigen-specific TCR ligand, MHC class I is an NK recognition structure and provides a "negative" signal to turn the NK cell "off " (Gumperz and Parham, 1995; Lanier and Phillips, 1996; Colonna, 1996; Moretta, et al., 1996). In humans, the natural killer cell inhibitory receptors (KIR) specifically recognize polymorphic sites in the HLA class I α1 domain. In mouse, the receptors on NK cells for MHC class I are the products of the Ly-49 multigene family (Yokoyama and Seaman, 1993). It has been directly demonstrated that Ly-49A is a class I MHC binding receptor (Karlhofer et al., 1992; Kane, 1994; Brennan et al., 1994). Ly-49 molecules are type II integral membrane proteins homologous to the C-type lectin family suggesting that ligands for Ly-49 could be carbohydrates (Yokoyama and Seaman, 1993; Gumperz and Parham, 1995). It has been shown by domain swapping experiments that the Ly-49 carbohydrate recognition domains are critically involved in Ly-49 binding to H-2 class I molecules (Brennan et al., 1996). Although the carbohydrate moieties of HLA class I do not seem to affect human KIR recognition (Gumperz et al., 1995), it has been shown that Ly-49A and Ly-49C can bind sulfated mono- and polysaccharides (Daniel et al., 1994; Brennan et al., 1995). In addition, a Ly-49 related rat molecule, NKR-P1, specifically expressed on NK cells, has been demonstrated to bind carbohydrates (Bezouska et al., 1994). Taken together, these results demonstrate a potential role of MHC class I oligosaccharide as a recognition structure of Ly-49 family members.

Some structural considerations

The three-dimensional crystal structures of MHC class I, TCR, and their complex have provided more direct information on the locations of glycosylation sites and how the oligosaccharide structure might affect or participate in the interaction. The MHC class I α1 and α2 domains form a peptide-binding groove with the α helices as sides and the β strands of the β sheets as the bottom (Bjorkman et al., 1987; Fremont et al., 1992). The *N*-linked glycans at residue 86 and 176 have interesting positions, in close proximity with the most variable region of the class I heavy chain which forms part of the peptide-binding groove (Bjorkman et al., 1987; Parham, 1996a). Recent crystal structures of TCR complexed with MHC/peptide have shown that the TCR contacts the MHC class I complex diagonally and the glycosylation sites are just outside the TCR contact sites (Garcia et al., 1996a; Garboczi et al., 1996). Although the MHC class I oligosaccharide structures were suggested to be pointing away from the TCR contact sites (Bjorkman et al., 1987), there is, in fact, no information on their orientation. In addition, oligosaccharide structures themselves are flexible (Bush, 1992) and could be large in size (Wier and Edidin, 1988). The possibility of MHC class I oligosaccharides interfering with TCR/MHC interaction is therefore intriguing (Parham, 1996b).

H. T Cell Activation

Generally, T cell activation can be considered as a two-step process: antigen recognition and signal transduction. Once the TCR recognizes the peptide/MHC complexes, these recognition events are translated to appropriate biochemical signals leading to T cell proliferation, differentiation and effector functions including cytokine production and cytotoxicity. These signaling events are also essential for T cell maturation in the thymus (Howe and Weiss, 1995). T cell activation is an extremely complicated yet poorly understood process involving multiple cell surface ligand pair interactions between the T cell and APC or target cells, and cascades of signaling events (Weiss and Littman, 1994).

Biochemical events during TCR signaling

1. Phosphorylation by protein tyrosine kinases

The earliest detectable event after TCR engagement is the tyrosine phosphorylation of several proteins (Weiss and Littman, 1994). The proteins include the TCR signaling components, most importantly, CD3 ε and ζ chains. Because the cytoplasmic tails of TCR α and β chains are too short for signal transduction, and the invariant CD3 chains and ζ chains do not contain intrinsic enzymatic activities, TCR signaling through the TCR complex must be mediated by other proteins. The CD3 ε chain and ζ chains have a consensus sequence termed immunoreceptor tyrosine-based activation motif (ITAM), which contains two tyrosine residues that are phosphorylated upon TCR stimulation by the protein tyrosine kinase (PTK), $p56^{lck}$ and/or $p59^{fyn}$. Both $p59^{fyn}$ and $p56^{lck}$ are src-family tyrosine kinases which typically possess a kinase domain, a src homology (SH) 2 domain and a SH3 domain.

The kinase activity is regulated by phosphorylation of their regulatory tyrosine residues. For instance, p56^{lck} has two tyrosine sites that are involved in the regulation of its kinase activity. Phosphorylation of Tyr394 is important for the activation of this enzyme while Tyr505 is a negative regulatory site, i.e. phosphorylation of this residue inhibits the activity of p56^{lck} kinase. It has been shown that a membrane tyrosine phosphatase, CD45, removes the phosphate group at Tyr505 of p56^{lck} upon TCR engagement, hence activating p56^{lck} (Ostergaard et al., 1989). The phosphorylated ITAM of the ζ chain subsequently associates with ZAP-70, a 70 kD PTK which is distinct from the src family of kinases. ZAP-70 has two SH2 domains, but no SH3 domains, and has no negative regulatory sites. ZAP-70 associates with the phosphorylated ITAM motif of the ζ chain through its SH2 domains. After ZAP-70 binds the ζ chain, the proximal src family kinase p56^{lck} or p59^{fyn} can phosphorylate ZAP-70 which modulates its kinase activity. It is noteworthy that the function of these signaling molecules depends both on their intrinsic kinase activity and their SH2 or SH3 domains which can serve as adapters to recruit other molecules into the TCR signaling complex (Xu and Littman, 1993; Pawson, 1995; Howe and Weiss, 1995).

2. Inositol lipid metabolism

The TCR is able to regulate the activation of the phospholipase C (PLC) signaling pathway. PLC regulates the hydrolysis of a membrane phospholipid called phosphatidylinositol-4,5-biphosphate (PIP_2) and produces diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). IP_3 binds its receptor on the ER and causes the release of intracellular calcium stores, and subsequently the influx of extracellular calcium. Increased calcium concentration is important for the function of a number of calcium-dependent enzymes. DAG is an activator of protein kinase C (PKC), a serine/threonine kinase whose substrates have not been well characterized.

Another TCR signaling pathway that affects lipid metabolism involves a lipid kinase called phosphatidylinositol 3-kinase or PI 3-kinase (Cantrell, 1996). PI 3-kinase consists of an 85 kD regulatory subunit which contains two SH2 domains and one SH3 domain, and a 110 kD catalytic subunit. This enzyme specifically phosphorylates the D-3 position of the inositol ring of phosphatidylinositol generating PI 3,4-P2, and PI 3,4,5-P3. An increase of these metabolites is observed after TCR stimulation, however the exact mechanism involved in PI 3-kinase activity during T cell activation is still unclear. PI 3-kinase is also intimately involved in CD28-mediated cosignaling (Rudd et al., 1994).

3. Ras-MAP kinase involvement

It has been demonstrated that the G protein, Ras, is activated upon TCR stimulation (Rudd et al., 1994; Cantrell. 1996). Activation of Ras depends on the ratio of bound GTP to GDP. Stimulation of TCR leads to the phosphorylation of the ITAM of the ζ chain. The SH2 domain-containing adapter protein, SHC, bind to the phosphorylated ITAM. SHC may associate with another adapter protein, Grb-2, which contains one SH2 domain and two SH3 domains. Sos is a guanine nucleotide releasing protein capable of exchanging GDP with GTP, and is associated with the Grb-2 SH3 domains via its proline-rich region. This results in the activation of Ras, and Ras activates the downstream mitogen-activated protein kinases (MAP kinases). MAP kinases are serine/threonine kinases and play an important role in the induction of several transcription factors in T cell activation.

It should be noted that T cell activation is not a simple all-or-none phenomenon, but instead, is a continuum of T cell signaling events and responses including tyrosine phosphorylation, PI hydrolysis, calcium flux, cytokine secretion, proliferation and cytotoxicity (Evavold et al., 1993; Sloan-Lancaster and Allen, 1996). The type of T cell responses achieved, to a great extent, depend on the type of TCR ligands engaged and co-stimulators involved. Based on the outcome of T cell stimulation, TCR ligands (MHC/peptide complexes) are classified into three categories: agonist, partial agonist and antagonist (Evavold et al., 1993). Agonist ligands induce full T cell responses, while antagonist ligands actively prevent or inhibit T cell activation triggered by an agonist. A partial agonist triggers a subset of T cell signaling events and some T cell responses (Sloan-Lancaster and Allen, 1996).

Coreceptor and accessory molecules in T cell activation

Usually engagement of TCR alone is not sufficient to fully activate T cells, especially under physiological conditions where TCR affinity for MHC complex is low and only a limited number of specific TCR/MHC complexes are involved. Participation of the coreceptor or accessory molecules is critical for T cells to achieve optimal functionalities. The interactions between the coreceptor or accessory molecules on T cells with their respective ligands on APC or target cells optimize or enhance T cell responses by the following mechanisms. 1) To increase the avidity between the T cell and APC or target cells, so the interaction between the TCR and MHC/peptide complex will be of sufficient avidity and duration for optimal TCR signaling. Most coreceptor and accessory molecules can function as this type of avidity enhancer. 2) To amplify the TCR initiated signal(s). In this case, the coreceptors and accessory molecules generate signals that are redundant or overlapping with the TCR initiated signals. 3) To transduce distinct signals or co-signals. The discovery of so many signaling molecules and the extensive cross-talk among different signaling pathways make the distinction between 2) and 3) less clear. It is likely that some accessory molecules may function through a combination of these mechanisms.

I. Role of CD8 in CTL Recognition and Activation

CD8 structure

CD8 consists of two disulfide-linked polypeptide chains, α and β, and exists either as an αα homodimer or αβ heterodimer. Both CD8 α and β are encoded by two distinct, but closely linked genes located on chromosome 6, and are members of the Ig superfamily. In mouse, the CD8 α chain (also called Lyt-2) is a 38 kD glycoprotein, while the CD8 β chain (also called Lyt-3) is a 28 to 30 kD polypeptide (Parnes, 1989). Although they share only limited ~30% amino acid sequence homology, the overall organization of CD8 α and β chains is similar. They both consist of an extracellular Ig-like domain, a membrane-proximal stalk (connecting peptide), a transmembrane region and a cytoplasmic tail. Both the mouse and human CD8 α chain cytoplasmic tail is serine/threonine phosphorylated, but its significance in CD8 fuction is unclear (Acre et al., 1987; Blue et al., 1987; Williams et al., 1991). The CD8 α chain cytoplasmic tail has a cysteine-containing motif, CXCP, which is the binding site for p56^{lck}, however, the CD8 β chain lacks this binding motif and is unable to associate with p56^{lck}. In mouse, a differentially spliced form of the CD8 α, called CD8 α', exists. The CD8 α' has a shortened cytoplasmic tail, only three amino acid long, which is unable to associate with p56^{lck} (Zamoyska and Parnes, 1988, Zamoyska et al., 1989). The α' chain preferentially forms dimers with β chain and is only expressed on the surface of immature thymocytes. It is not known whether this CD8 α'β heterodimer plays a role in T cell thymic maturation.

CD8 is expressed on mouse thymocytes and at the mature T cell surface primarily as a disulfide-linked αβ heterodimer. In human, although the majority of $CD8^+$ T cells express αβ heterodimers, some $CD8^+$ T cells do express CD8 αα homodimers. It is believed that CD8 β chains are not expressed on T cell surfaces alone, because the expression of CD8 β chain requires the concomitant expression of CD8 α (Blanc et al., 1988).

CD8 in CTL recognition, activation and effector function

Based on anti-CD8 antibody blocking experiments, Swain (1983) proposed that CD8 plays an important role (albeit not always obligatory) in T cell responses to MHC class I antigen by serving as a "facilitator" interacting with class I molecules. More direct evidence came from CD8 gene transfer experiments. Introduction of CD8 genes into T cell hybridomas enhances T cell cytolytic activity (Dembic et al., 1987) as well as IL-2 production (Gabert et al., 1987; Wheeler et al., 1992). These functional data clearly indicate that CD8 is actively involved in T cell activation. It has also been shown that CD8 transfectants (Norment et al., 1988) and CD8-incorporated liposomes (Rosenstein et al., 1989) bind to class I expressing cells or vesicles, directly demonstrating that CD8 binds MHC class I molecules.

Further studies have been carried out to understand how CD8 interacts with MHC class I molecules. Several lines of evidence indicate that the highly conserved acidic loop in the α3 domain of MHC class I is critically involved in CD8 binding. In both mouse and human, the loop including residues 222 to 229 in the α3 domain contains four negatively charged amino acids, glutamic acid or aspartic acid. Potter and colleagues have found that the substitution of any of these acidic amino acids with a lysine abrogated or greatly reduced the CD8-dependent CTL lysis of targets expressing these mutant molecules (Potter et al., 1987; Connolly et al., 1988; Potter et al., 1989). Synthetic peptides corresponding to the acidic loop of HLA class I molecules blocks T cell differentiation *in vitro* (Clayberger et al., 1994). Transgenic mice expressing a Glu227->Lys α3 domain mutant class I failed to eliminate self-reactive T cells, suggesting a role of MHC class I α3 domain interaction with CD8 in thymocyte negative selection processes (Ingold et al., 1991). In addition to these informative functional analyses, a role for the acidic loop of the α3 domain of class I in mediating CD8-class I binding has also been established. Cell-cell binding assays combined with site-directed mutagenesis have shown that human CD8 αα homodimer-expressing cells failed to bind cells expressing HLA class I with substitutions of the acidic amino acids in the acidic loop in the α3 domain, directly demonstrating that the negatively charged loop (residue 222 - 229) in the α3 domain of MHC class I molecules is a binding site for CD8 (Salter et al., 1989 & 1990).

Several amino acids of CD8 involved in the interaction with MHC class I have been identified. Loops extending from the CD8 Ig-like variable domains are important sites for MHC class I interactions as shown by site-directed mutagenesis and cell-cell adhesion assays (Sanders et al., 1991). The crystal structure of a soluble form of the human CD8 αα homodimer reveals that these sites are generally electrostatically positive (Leahy et al., 1992), which favors the interaction with the negatively charged α3 acidic loop of MHC class I. It has been reported that a negatively charged region in the α2 domain of MHC class I may also be involved in the interaction with CD8 (Giblin et al., 1994; Sun et al., 1995).

Mechanisms of CD8 participation in CD8-dependent CTL activation

Traditionally, CD8 dependency is operationally defined by the ability of anti-CD8 antibody to inhibit the $CD8^+$ T cell responses. However, the biochemical and biophysical basis for CD8 dependency is not well understood. One explanation is that because the TCR usually has a low affinity for the MHC and the number of MHC/antigenic peptide complexes is low, CD8 is usually required to facilitate these interactions (Alexander et al., 1991; Takeshita et al., 1993; Cai and Sprent, 1996). How CD8 participates in CTL recognition and activation has been under intensive study. It is believed that CD8 has dual functions during the T cell activation process (Fig. 1-1). The CD8 molecule can serve as a coreceptor in that it binds to the same MHC class I molecule as the TCR to facilitate efficient TCR/MHC interactions and possibly signal transduction (Potter et al., 1987; Julius et al., 1993; Miceli and Parnes, 1993). In addition to serving as a coreceptor, CD8 molecules can be "activated" or "triggered" by TCR stimulation to bind to MHC class I molecules with high avidity, including class I not recognized by the TCR as antigenic complexes (i.e. non-Ag class I), to augment CTL responses and thus serve an accessory molecule function (Fig. 1-1) (O'Rourke et al., 1990; O'Rourke and Mescher, 1993; Kane and Mescher, 1993; Takeshita et al., 1993; Luescher et al., 1995).

1. CD8 as a coreceptor

CD8 must bind to the same antigenic peptide/MHC class I complex as the TCR does to function as a coreceptor. This conclusion is inferred from the following observations. If CD8 can function as an adhesion (or accessory) molecule to bind to MHC class I [including those not engaged by specific TCR (non-Ag class I)], then the participation of non-Ag class I alone should be able to enhance CTL responses. Several groups have found that the antigenic MHC class I (being peptide antigen) with an $\alpha3$ domain mutation rendering it "non-CD8 binding" as demonstrated by static-state cell-cell binding assays, was unable to trigger CTL cytotoxicity even in the presence of non-Ag class I which is not a TCR ligand (Potter et al., 1989; Salter et al., 1990). The natural conclusion is that CD8 must bind to the same MHC class I that TCR binds, hence serving as a coreceptor with the TCR. More recently, by using photoaffinity labeling and surface plasmon resonance techniques, it has been directly demonstrated that CD8 can serve as a coreceptor by strengthening cell surface or membrane-bound TCR binding to soluble MHC class I molecules, reinforcing the conclusion that CD8 can function as a coreceptor (Luescher et al., 1995; Garcia et al., 1996b).

2. CD8 as an accessory molecule

Direct binding between CD8 transfectants or CD8-containing liposomes and class I-expressing cells or vesicles has been demonstrated (Norment et al., 1988; Rosenstein et al., 1989), indicating that CD8 can function as an adhesion or accessory molecule. Also from these experiments, it is appreciated that this type of interaction is inefficient. The static-state CD8 binding to MHC class I is of very low affinity because overexpression of CD8 is required to achieve even a low level of adhesion. The work pioneered by Mescher and co-workers has extended these findings by showing that efficient, high avidity CD8 binding to class I is achieved by TCR stimulation (O'Rourke et al., 1990; O'Rourke and Mescher, 1993). The avidity-enhanced CD8 binding occurs even when the MHC class I ligand is not recognized by TCR (non-Ag class I). This activation-dependent adhesion is termed TCR-activated or triggered binding (Springer, 1990), and has also been demonstrated for other adhesion molecules binding to their respective ligands, including LFA-1 to ICAM-1,2,3 (Dustin and Springer, 1989; Ybarrondo et al., 1995), VLAs to extracellular matrix proteins (Shimizu et al., 1990), and CD2 to LFA-3 (Moingeon et al., 1989). Importantly, similar to the above mentioned adhesion molecules, the activated CD8 binding to MHC class I molecules enhances CTL function as demonstrated by increased CTL degranulation, presumably by delivering additional signal(s) (O'Rourke et al., 1990; O'Rourke and Mescher, 1993). It has been shown that TCR signaling events, particularly PTK activity and intact cytoskeletal function, are necessary for activated CD8 binding to MHC class I (O'Rourke et al., 1990 & 1991; O'Rourke and Mescher, 1994). The exact mechanism(s) of how CD8 mediates avidity-enhanced binding to MHC class I is still unclear, but it is not due to an upregulated CD8 expression on CTL after TCR stimulation (O'Rourke et al., 1990). Non-exclusive explanations for activated CD8 binding may include 1) aggregation or clustering of CD8 to the CTL-target contact site due to cytoskeleton reorganization triggered by TCR engagement (Kinch et al., 1994); or, 2) conformational changes following TCR stimulation, analogous to that observed for LFA-1, resulting in increased affinity of CD8 (Cabanas and Hogg, 1993).

3. CD8 as a signaling molecule

CD8α and CD4 have been reported to be associated with a protein tyrosine kinase, $p56^{lck}$ (Veillette et al., 1988). Original studies have shown that cross-linking of CD4 or CD8 induces $p56^{lck}$ kinase activity (Veillette et al., 1989; Lou and Sefton, 1990). This has led to speculation that, in addition to its adhesive role, CD8 and CD4 may also play a signaling role during CTL activation via their associated $p56^{lck}$. About 5 to 10% of the total cellular $p56^{lck}$ has been shown to be associated with CD8, while up to 75% of cellular $p56^{lck}$ is associated with CD4 (Julius et al., 1993). Further attempts to demonstrate a role of CD8-$p56^{lck}$ association in CTL maturation and activation have produced conflicting results. When genetically engineered CD8α, that does not associate with $p56^{lck}$, was transfected into T cell hybridomas, it was unable to augment T cell responses, demonstrating that CD8 association with $p56^{lck}$ is required for CD8 function (Zamoyska et al., 1989; Letourneur et al., 1990; Miceli et al., 1991; Hoeveler and Malissen, 1993). Another approach is to use CD8α gene-disrupted mice. In these mice, $CD8^+$ T cell development is greatly impaired. However, introduction of a CD8 α transgene construct that is unable to associate with $p56^{lck}$ into the CD8α gene-disrupted mice was able to rescue $CD8^+$ T cell maturation (Levin et al., 1993; Chan et al., 1993; Fung-Leung et al., 1993; Smith et al., 1996). Thus, in this system, an essential role of the CD8α cytoplasmic tail or its association with $p56^{lck}$ for thymic selection has not been demonstrated. The apparent differential requirements of T cell hybridomas and thymocytes for CD8-associated $p56^{lck}$ could be due to the different requirements for signaling in immature versus mature T cells. In summary, it is likely that CD8 enhances CTL responses by several mechanisms, including signaling. If signaling is involved, it may not be solely dependent on its associated $p56^{lck}$ or $p56^{lck}$ kinase activity (Xu and Littman, 1993; Pawson, 1995).

O'Rourke and co-workers have found that soluble anti-TCR mAb-activated CD8 binding to non-Ag class I can enhance T cell phosphoinositide hydrolysis and calcium flux (O'Rourke and Mescher, 1992 & 1994). The activated CD8 binding is inhibitable by PTK and PKC inhibitors, suggesting signaling events are involved. Along the same line, Anel et al. (1996) further showed increased tyrosine phosphorylation of several proteins and decreased tyrosine phosphorylation of $p56^{lck}$ upon activated CD8 binding to class I. Interestingly, total cellular $p56^{lck}$ activity increased after CD8 binding to class

I. These results suggest that activated binding to MHC class I could mediate additional signaling for optimal CTL activation.

Role of the CD8 β chain in CD8⁺ T cell recognition

Early investigations about the role of CD8 on CTL maturation and functions have concentrated on CD8 αα homodimers, mainly because CD8 αα homodimers are functionally competent. The CD8 molecule is typically expressed as a disulfide-linked αβ heterodimer on mature murine and human CD8⁺ T cells (Miceli and Parnes, 1993); however, the relative contributions of CD8 α and β chains to T cell development and activation have not been fully determined (Zamoyska, 1994). It has been shown by gene transfection and gene disruption technologies that CD8 αβ heterodimers can further enhance T cell responses and promote CD8⁺ T cell maturation relative to that observed with CD8 αα homodimer expression alone (Wheeler et al., 1992; Nakayama et al., 1994; Fung-Leung et al., 1994; Crooks and Littman, 1994), suggesting a role of CD8 β chain in CD8⁺ T cell function. The improved CD8 αβ heterodimer function over the CD8 αα homodimer can be at least attributed to more efficient CD8 coreceptor function (Luescher et al., 1995; Renard et al., 1996). Although CD8 β chain itself does not associate with $p56^{lck}$, it can modify the association and activity of CD8 α chain-associated $p56^{lck}$ and contribute to CTL activation (Irie et al., 1995) .

J. Rationale

Although tremendous progress has been made in the molecular mechanisms of CTL recognition and activation, unsolved issues remain. MHC class I structural requirements for TCR and CD8 recognition are not fully elucidated, nor is it fully determined what effects MHC class I post-translational modifications have on T cell recognition. MHC class I is a glycoprotein, however, the potential roles of glycosylation of MHC class I molecules in CTL recognition and activation is not well understood. CD8 has dual functions, i.e. coreceptor and accessory function, and both are important during CTL activation. However, the relative contribution of the two functions to T cell activation is less clear. Furthermore, the structural requirements of MHC class I to effectively serve as CD8 coreceptor and accessory ligands require additional elucidation. To directly address these issues, I took a reductionist's approach and examined CTL interactions with various natural and mutated purified MHC class I molecules in a solid phase system. In this system, the immunoaffinity purified MHC class I molecules can be immobilized on the plastic and the density of the class I molecules can be precisely controlled. The purified MHC class I molecules can also efficiently form MHC class I/peptide complexes in the plastic wells to effectively trigger antigen-specific CTL responses. Molecular interactions between TCR, MHC class I and CD8 can therefore be examined to the exclusion of the potential participation of other accessory ligands. In addition, coimmobilization of antigenic and non-antigenic MHC class I molecules can be readily achieved, providing a means to dissect CD8 coreceptor and accessory functions. The overall goal of this study is to further understand the roles and mechanisms of MHC class I and CD8 interaction in CTL recognition and activation.

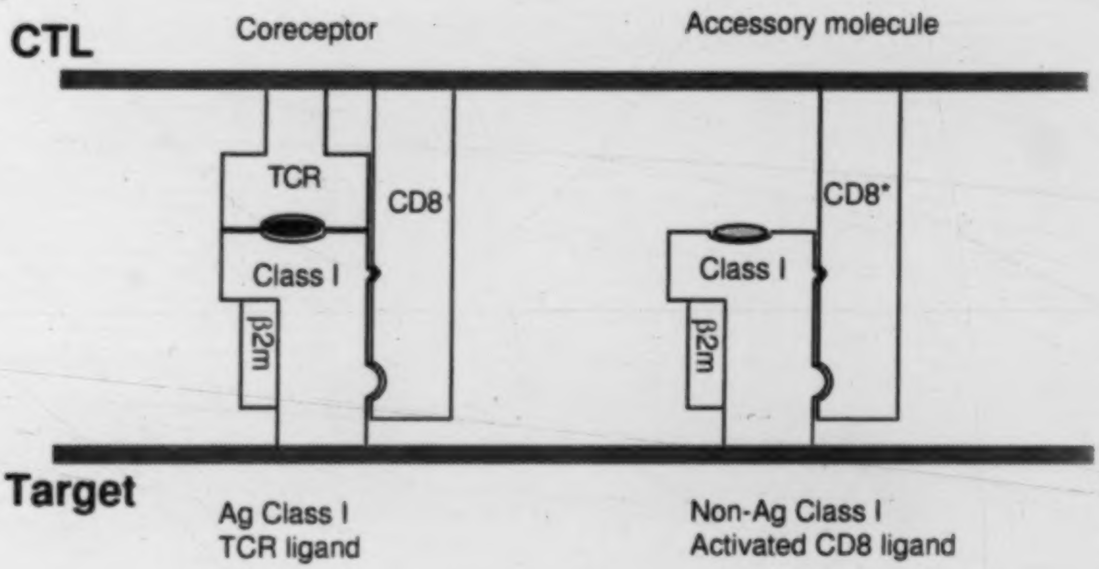

CTL
Coreceptor
Accessory molecule
TCR
CD8
Class I
β2m
CD8*
Class I
β2m
Target
Ag Class I
TCR ligand
Non-Ag Class I
Activated CD8 ligand

Figure 1-1. CD8 interactions with MHC class I molecules. CD8 serves dual functions during the T cell activation process. The CD8 molecule can serve as a coreceptor in that it binds to the same MHC class I molecule as the TCR to facilitate efficient TCR/MHC interactions (*left*). In addition to serving as a coreceptor, CD8 molecules can be "triggered" by TCR stimulation to bind to MHC class I molecules with high avidity, including class I not recognized by the TCR as antigenic complexes (i.e. non-Ag class I), to augment CTL responses and thus serve an accessory molecule function (*right*).

CHAPTER II

DIFFERENTIAL ABILITY OF H-2 K^b SUBSETS TO SERVE AS TCR LIGANDS FOR ALLO-CTL RECOGNITION: POTENTIAL ROLE OF *N*-LINKED GLYCOSYLATION

(Most of the data presented in this chapter are reproduced from **The Journal of Experimental Medicine, 1995, 181, 1773- 1784.** by copyright permission of The Rockfeller University Press.)

A. Introduction

Class I MHC-restricted $CD8^+$ CTL recognize a complex of MHC class I heavy chain, β_2-microglobulin (β_2m) and a peptide antigen. An allo-specific $CD8^+$ CTL clone usually recognizes complexes of allo-class I and one or only a small number of specific endogenously processed peptide(s) (Heath et al, 1989 & 1991; Rotzschke et al, 1991, Udaka, 1992). Collectively, a population of allo-specific CTL respond to allo-class I and a diverse array of endogenous bound peptides (Sherman and Chattopadhyay, 1993). Activation of CD8-dependent CTL requires MHC complex interaction with the TCR, and participation of CD8 which facilitates TCR/class I interactions (Garcia et al., 1996), mediates adhesion (O'Rourke et al., 1990; Kane and Mescher, 1993; O'Rourke and Mescher, 1993; Luescher et al., 1995) and/or provides cosignalling functions (O'Rourke and Mescher, 1993; Anel et al., 1996).

It has been shown previously that immobilized purified class I MHC alloantigens serve to trigger several allo-specific $CD8^+$ CTL clones and therefore class I alloantigen is a necessary and sufficient ligand for CTL adhesion and activation of a degranulation response (Kane et al., 1989b). When MHC class I is the only ligand on the surface, the CTL adhesion and response depends on the participation of both TCR and CD8 (Kane and Mescher, 1993). Although specific bound endogenous peptides are required for allo-CTL recognition, it is not known whether all potential forms of a given allo-class I molecule are capable of serving as alloantigens even when bound with relevant peptide(s).

Murine MHC class I molecules have 2 or 3 *N*-linked glycans with K^b usually possessing two *N*-linked complex-type glycosylations at Asn-86 and Asn-176 of the α_1 and α_2 domains, respectively (Klein, 1986). It has been clearly demonstrated that the same class I allele products can be expressed at the cell surface with heterogeneous *N*-linked carbohydrates (Jones, 1977; Gilmer, 1982; Le and Doyle, 1982; Swiedler et al., 1985). However, the composition, structure and sequence of the carbohydrate modifications is not well establised. The removal of sialic acids has reduced, although not eliminated, the size and charge heterogeneity of class I (Parham et al., 1974; Jones, 1977; Krakauer, et al., 1980; Gilmer, 1982; Le and Doyle, 1982; Swiedler et al., 1985), suggesting that sialylation contributes at least partly to the observed microheterogeneity. Other potential contributing factors may include phosphorylation (Gilmer, 1982; Thor et al., 1993), sulfation and oligosacchride structure branching patterns (Swiedler et al., 1983 & 1985)

The exact role(s) of MHC class I glycosylation on CTL recognition and activation is not well understood. Earlier studies indicate that oligosaccharides expressed on MHC class I are not required for allo-CTL recognition. Enzymatic removal of *N*-linked glycosylation on purified murine class I K^k molecules did not affect its ability to induce an allo-CTL response (Goldstein and Mescher, 1985). Abrogation of *N*-linked glycosylation acceptor sites on L^d by site-directed mutagenesis (Shiroshi et al., 1985; Miyazaki et al, 1986) altered L^d recognition by several specific mAbs, but did not affect the allo-CTL lysis of the transfectants expressing these mutant molecules. However, it is unclear what influence the complexity of *N*-linked glycosylation on class I may have on CTL recognition and effector functions (Powell et al., 1985; Degen et al., 1989), although cell surface sialylation (Gilmer et al., 1982; Powell et al., 1987a; Boog et al., 1989), possibly on MHC class I, influences class I antigen presentation. It is the purpose of this study to directly address this complementary issue.

B. Materials and Methods

mAbs. M1/42, a rat anti-mouse mAb (IgG_{2a}) reactive with most H-2 class I antigens (Springer, 1980) was obtained from American Type Culture Collection (ATCC). The K^b-specific mAbs used in this study were: Y3 (IgG_{2b}) (Hammerling et al., 1982); B8-24-3 (IgG_1) (Ajitkumar et al., 1988); AF6 (IgG_{2a}) (Loken and Stall, 1982) obtained from ATCC; and 5F1 (IgG_{2b}) (Sherman and Randolph, 1981), a gift from Dr. L. Sherman, Research Institute Scripps Clinic, La Jolla, CA; and 100-30 (IgG_{2b}) (Lemke et al., 1979), a gift from Dr. U. Hammerling, Memorial Sloan-Kettering Cancer Center, New York, NY. The S19.8 (IgG_{2b}), an anti-mouse β_2m (Ly-m11 epitope) mAb which specifically recognizes β_2m of *b* strain mice (Tada et al., 1980; Margulies et al., 1983), was also a gift from Dr. U. Hammerling. A hamster mAb that recognizes CD3ε, 145-2C11 (Leo et al., 1987), was obtained from ATCC. The anti-CD8 mAb, 3.168 (IgM) (Sarmiento et al., 1980), was a gift from Dr. A. O'Rourke, Research Institute Scripps Clinic, La Jolla, CA.

Enzymes. Recombinant *Streptomyces plicatus* Endoglycosidase H (Endo H) was purchased from Boehringer Mannheim Biochemica and Endoglycosidase F (Endo F) purified from *Flavobacterium meningosepticum* was purchased from

Du Pont Company, Wilmington, DE. Type V neuraminidase (NANAse) from *Clostridium perfringens* was purchased from Sigma Chemical Co., St. Louis, MO.

Tumor cell lines. The murine T cell lymphoma cell line EL4 (H-2^b) and myeloid leukemia cell line C1498 (H-2^b) were obtained from ATCC. The murine lymphoma cell line RMA (H-2^b) was kindly provided by Dr. W. Jefferies, University of British Columbia, Vancouver, Canada. ANA-1, a macrophage cell line of C57BL/6 origin (Blasi et al., 1987), was a gift from Dr. M. Belosevic, University of Alberta, Edmonton, Canada. The murine pre-B cell line, R8 (H-2^{bxd}) (Tatake and Zeff, 1993) was provided by Dr. R. Zeff, University of Connecticut, Farmington, CT. The human transfected cell line T2K^b (Wei and Cresswell, 1992) was a gift from Dr. P. Cresswell, Yale University, New Haven, CT.

Cloned CTL. The K^b allo-specific CTL clones 11, 35, 6, 13 and AB.1 have been well documented (Kane et al., 1988 & 1989b). Clone AB.1 was derived from limiting dilution culture of a Balb/c (H-2^d) anti C57BL/6 (H-2^b) mixed lymphocyte culture. Clone 6, 11, 35 were derived from peritoneal exudate cells of (B10.BR X B10.D2)F_1 (H-2^k X H-2^d) mice 10 to 13 days after priming by intraperitoneal injection with 2 X 10^7 EL4 cells. Cells were stimulated *in vitro* under limiting dilution conditions with irradiated B10.A(5R) (H-2^{i5}) spleen cells and 10% rat Con A supernatants. Clone 13 was generated in the same way as clone 6, 11, and 35, but from a B10.D2 (H-2^d) mouse (Heath et al., 1991). All the K^b-specific clones were stimulated weekly with irradiated C57BL/6 spleen cells. The D^d allo-specific CTL clone 10/1 was derived from C57BL/6 spleen cells primed *in vitro* with irradiated (3,000 rad) Balb/c spleen cells and cloned one week later by limiting dilution in the presence of 5% Con A supernatant. Clone 10/1 was stimulated weekly with irradiated Balb/c spleen cells. All the allo CTL clones were cultured in 10% FCS-RPMI 1640 supplemented with 20 mM Hepes, 2 mM L-glutamine, 5 x 10^{-5} M 2-ME, 100 µg/ml penicillin-streptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 15 U/ml recombinant human IL-2.

Isolation of H-2 K^b subsets. The H-2 K^b was purified from various cell lines by immunoaffinity chromatography as described (Kane et al., 1989a) with modifications. EL4 cells were propagated *in vivo* as ascites. Usually, 1.5 - 2.0 x 10^{10} EL4 cells were harvested from 30 C57BL/6 (H-2^b) retired breeder mice one week after i.p. injection of 20 x 10^6 cells. The ANA-1, RMA, R8 and C1498 cells were grown in 5% FCS-DMEM and 1.0-1.5 x 10^{10} cells were harvested for K^b purification.

Typically, cells were lysed by nitrogen cavitation, centrifuged at low speed to remove the nuclei and then at high speed to pellet membranes (Mescher et al., 1983). These crude membranes were solubilized in 1% Triton X-100, 20 mM Tris containing 2 mM PMSF. The detergent lysates were then passed over a Y3 affinity column preceded by a Sepharose 4B precolumn.

After the lysate had passed over the columns, the precolumn was disconnected and the Y3 column was washed sequentially with 10-20 column volumes of each of the following buffers: 1) 0.5% Triton X-100, 10 mM sodium phosphate, pH 7.2; 2) 0.5% Triton X-100, 10 mM Tris, 40 mM NaCl, pH 8.0; and 3) 0.1% deoxycholate (DOC), 10 mM Tris, 40 mM NaCl, pH 8.2. No immunoreactive K^b was eluted off the column from these washes as detected by solid-phase ELISA.

Two sequential elutions were performed immediately after the column washes. The first elution buffer, 0.5% DOC, 0.65 M NaCl, 10 mM Tris, pH 8.3-8.5, has been described previously (Kane et al., 1989a). Following elution with this buffer, a second elution is done using a buffer of higher pH: 0.5% DOC, 0.15 M NaCl, 15 mM Na_2CO_3, pH 10.5. For some preparations, fractions of the first (pH 8.5) or second (pH 10.5) elution showing strong ELISA reactivity with the K^b-specific mAb Y3 as first antibody were separately pooled and stored at -20°C. Usually, the material was used within six months of storage. In some preparations, only a single pH 10.5 elution buffer was applied.

Immunoaffinity purifications of other murine MHC molecules. H-2 K^k molecules were purified from RDM-4 (H-2^k) cells using a 11-4.1 immunoaffinity column (Mescher et al., 1983). The I-E^d, I-A^d molecules were purified from A20 (H-2^d) cell lysates using MK-D6 and 14-4-4s affinity columns, respectively (Kane, 1994).

^{125}I labeling of EL4 cells. For monitoring the purification of cell surface K^b molecules, 20 x 10^6 EL4 cells were surface iodinated and their lysates added to the routine EL4 preparation as tracer. Lactoperoxidase/oxidase-catalysed iodination of EL4 cells was performed following the procedure described by Trowbridge et al. (1975). Briefly, 20 x 10^6 EL4 cells were washed three times with D-PBS and resuspended in 1 ml 5 mM glucose-PBS. The cell suspension was added along with 1 mCi $Na^{125}I$ (ICN Biochemicals, Costa Mesa, CA) and 100 µl oxidase/lactoperoxidase mixture (containing 7.25 U of lactoperoxidase and 0.25 U of glucose oxidase), and incubated at RT for 10 min. The EL4 cells were spun down and washed three times with D-PBS at 4°C. Then EL4 cells were lysed in 1% Triton X-100, 20 mM Tris, 2 mM PMSF, pH 7.2 on ice for 40 min. The nuclei were pelleted by centrifuging in a microcentrifuge at setting "14" for 5 min. The supernatant were added to the EL4 cell lysate and loaded onto the Y3 column.

Immobilization and coimmobilization of purified MHC proteins on plastic. The purified class I or class II molecules were directly diluted into D-PBS, pH 7.4, at a ratio of 1:20 or higher to minimize the effect of DOC on protein binding. For coimmobilization experiments, two purified MHC class I proteins were diluted into D-PBS at desired densities. A volume of 100 µl of diluted samples were placed into Falcon MicroTest III flexible 96-well microtiter plate (Becton Dickinson, Oxnard,CA) and incubated for 1.5 h at RT. Then the plate wells were washed three times with D-PBS and blocked with 1% BSA-PBS or 2% FCS-PBS for 30 min at RT.

ELISA of immobilized K^b molecules. Direct ELISA of affinity-purified H-2 K^b was performed as previously described (Kane et al., 1989a). Briefly, eluted K^b fractions or pooled samples were immobilized on plastic wells and blocked with 2% FCS-PBS. The K^b-specific first antibody was added and incubated for 45 min on ice. The first antibodies used were either purified by ammonium sulfate precipitation or as culture supernatant. Unbound first antibody was removed by three washes with D-PBS. Horseradish peroxidase (HRP)-conjugated goat anti-rat or rat anti-mouse IgG second antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) was added and incubated for 45 min on ice. After six washes with PBS, the ELISA reaction buffer consisting of 0.05 M Na_2HPO_4, 0.025 M citric acid, 800 µg/ml *O*-phenylenediamine hydrochloride (Calbiochem, San Diego, CA) and 0.15% H_2O_2 was added. The reaction was stopped by addition of 2 N H_2SO_4 during the linear phase of color development. OD at 490 nm was determined using a microtiter plate reader (Molecular Devices, Mountain View, CA).

Assays of CTL binding and degranulation. CTL adhesion and degranulation responses to purified class I and coimmobilized proteins has been previously described in detail (Kane and Mescher, 1993). CTL were ^{51}Cr-labelled, washed, resuspended in assay medium (2% FCS-RPMI) and added to wells at 1 to 2 x 10^5 cells/well at a volume of 0.1 ml. Plates were centrifuged for 4 min at 400 rpm and incubated for 4 h at 37°C in a CO_2 incubator. At the end of the incubation, unbound cells were removed using a pipettor, the plastic well bottoms cut off, and the radioactivity determined in a γ-counter. Spontaneous release was determined for cells incubated under same conditions. Cell binding was calculated as: percent cell bound=100 x [(cpm bound)/(total cpm-spontaneous cpm)]. Experiments were done in triplicate for each condition unless specified.

Degranulation by the CTL was assessed by measuring the serine esterase (SE) activity released into the medium (Kane et al., 1989b; Kane and Mescher, 1993). Determination of CTL binding and SE release was done simultaneously from the same wells. At the end the of incubation and before removal of unbound cells, a 20 µl aliquot of supernatant was taken and mixed with 160 µl of reaction mixture. The reaction mixture contained 2 x 10^{-4} M *N*-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) (Calbiochem) and 2.2 x 10^{-4} M 5'5-dithiobis(2-nitrobenzonic acid) (Sigma Chemical Co., St. Louis, MO) in PBS. The SE release assay was allowed to proceed for 20 min and OD_{405nm} was determined using a plate reader. SE release is expressed as ΔOD_{405nm} = OD_{405nm}(CTL +immobilized class I) - OD_{405nm}(CTL +wells blocked with 2% FCS in PBS). Determinations were done in triplicate for each condition unless specified.

In experiments employing fluid phase anti-CD3 mAb, the antibody was added to the cells in suspension and the cells immediately placed into the well.

The anti-CD8 mAb blocking experiment was performed by incubating CTL with the antibody at the indicated concentration at RT for 30 min.

Acid extraction of peptides from purified K^b subsets. Peptides associated with purified K^b were extracted by trifluoroacetic acid (TFA) (Falk et al., 1991; Sherman et al., 1993). Peak fractions of pH 8.5-eluted (Ly-m11$^+$) and pH 10.5-eluted (M1/42^{high}) subsets were separately pooled. The pools were serially diluted, ELISA using Y3 as first antibody was performed and equivalent amounts of Y3-reactive K^b (defined as equal value of ELISA OD value x sample volume) were subjected to acid extraction with TFA. A 10% TFA-H_2O solution was added to the samples and a final concentration of 0.05% TFA (pH < 2.0) achieved. Because DOC precipitates when pH is below 8.0, the samples turned into slurry at this point. The slurry was gently vortexed and then rotated for 30 min at 4°C. The samples then were centrifuged at 5000 x g in Centricon-10 microconcentrators (Amicon Division, W. R. Grace & Co. Conn.), and the filtrates (< 10^4 mol wt) were collected and dried on a Savant SpeedVac Syetem (Savant Instruments, Farmingdal, NY). The dried samples were resuspended in 500 μl of 0.1% TFA-H_2O and separated on C_{18} reverse phase HPLC. Both pH 8.5 and pH 10.5 elution buffers were subjected to identical treatment in parallel as mock-treated controls.

HPLC separation of extracted peptides. Peptide were separated on a narrow-bore C_{18} (2 mm x 25 cm) column set up on a Beckman System Gold HPLC. The solvents were A: 0.1% TFA-H_2O; B: 0.09% TFA, 80% acetonitrile, 20% H_2O. A linear gradient of acetonitrile was applied with the gradient rate of 0.8% acetonitrile/min and a flow rate of 0.2 ml/min. Fractions corresponding to the 20-50% acetonitrile gradient were pooled, lyophilized, washed and reconstituted in 50 µl D-PBS for the sensitization of T2K^b cells for allo-CTL lysis.

Cytotoxicity assay. Peptides extracted from purified K^b were tested for their ability to sensitize T2K^b cells for lysis by allo-specific CTL clones. ^{51}Cr-labelled T2K^b cells were resuspended in assay medium at a density of 3 x 10^6/ml. A volume of 50 µl of the cell suspension was incubated with various dilutions of peptides isolated from purified K^b subsets at 37°C for 45 min. Then the T2K^b cells were diluted to 1 x 10^5/ml with assay medium and 100 µl was incubated with various CTL clones at a 5:1 E:T ratio for 5 h in V-bottom plates in triplicate. In all experiments, EL4 cells (H-2^b) were included as positive controls, while P815 or A20 cells (both H-2^d) were used as negative controls. Percent specific ^{51}Cr release was calculated as: [(experimental release - spontaneous release)/(maximum release - spontaneous release)] x 100. Results are expressed as mean % specific ^{51}Cr release ± SD.

SDS-PAGE. The purified H-2 K^b subsets were analyzed by 10% or 12.5% SDS-PAGE under reducing conditions (Laemmli, 1970). Gels were stained by Coomassie blue. Autoradiography was also carried out to visualize the ^{125}I-labelled proteins in the gel.

Endo H, Endo F and NANAse treatment of purified H-2 K^b subsets. The isolated H-2 K^b proteins (typically 60 µl) were mixed with 600 µl of cold acetone in the presence of 20 µg BSA as carrier. After standing at -20°C overnight, the precipitates were collected by centrifugation, dissolved and heated for 5 min in 50 µl of their respective enzyme incubation buffers (Williams et al., 1985) before addition of enzymes. For Endo H treatment, buffer consisting of 0.1

M sodium citrate, 0.1% SDS, and 0.2% 2ME, pH 6.0 was used. For Endo F treatment, buffer consisting of 0.1 M sodium phosphate, 33 mM EDTA, 0.1% SDS, and 0.2% 2ME, pH 6.0 was employed. Samples were incubated with 3 - 6 mU of Endo H or 0.5- 1.0 U of Endo F for 16 h at 37°C (Goldstein and Mescher, 1985; Williams et al., 1985). NANAse treatment of samples was performed with 0.5 - 0.6 U of NANAse at 37°C for 4 h in 0.1 M potassium acetate, pH 4.5 (Boog et al., 1989). In all the enzyme treatments, 10 μg aprotinin was included. The effectiveness of enzyme treatments were monitored by enzyme digestions of ribonuclease B carried out in parallel.

C. Results

Isolation of two serologically distinct K^b subsets from H-2^b-expressing cell lines. Standard protocols for immunoaffinity purification of murine class I MHC molecules from cell detergent lysates typically involve elution of the antigens from the affinity column at pH 8.5 in 0.5% DOC detergent (Mescher et al., 1983; Kane et al., 1989a). However, we have found that not all EL4 cell K^b is eluted from a Y3 mAb affinity column using standard elution conditions at pH 8.5. When an elution buffer of higher pH (pH 10.5) was used, two peaks reactive with K^b specific mAb, Y3, were usually observed (Fig. 2-1 A). This elution profile implied that there might exist two major subsets of K^b molecules. Separation of these two subsets by sequential elutions was attempted. Immediately following the pH 8.5 elution, a second immunoreactive K^b peak can be further eluted as determined by solid phase ELISA using Y3 as first antibody (Fig. 2-1 B). We also analyzed individual eluted fractions by ELISA with the S19.8 mAb which recognizes the Ly-m11 epitope on β_2m when associated with K^b (Tatake and Zeff, 1993; Tatake et al., 1995). S19.8-reactive material eluted coincidently with the first Y3-reactive peak (peak 1), but not the second (peak 2), indicating that two serologically distinct K^b subsets can be separately isolated from EL4 (H-2^b) using a Y3 affinity column (Fig. 2-1).

The two K^b subsets can also be isolated from various cell lineages including T cell lines, e.g. EL4 (Fig. 2-1) and RMA (Fig. 2-2 B); macrophage/monocyte lines, e.g. ANA-1 (Fig. 2-2 A); pre-B cell line, R8 (Fig. 2-2 C) and myeloid leukemia line, C1498 (Fig. 2-2 D). This suggests that the expression and isolation of two serologically different K^b subsets is not restricted to a particular cell line or lineage. In all cases, although the pH 8.5- and pH 10.5-eluted K^b are reactive with Y3 mAb, only the pH 8.5-eluted K^b molecules are recognized by S19.8 (Ly-m11) mAb (Fig. 2-1 and Fig. 2-2). It is noteworthy that, judging by Y3 mAb reactivity, the relative ratio of pH 8.5-eluted K^b versus pH 10.5-eluted K^b is variable. For example, the ANA-1 cell line contains more pH 8.5-eluted K^b, while the R8 and C1498 cell lines contain much less (Fig. 2-2). To further characterize the two K^b subsets, both serologically and functionally, we have gone on to use K^b subsets isolated from EL4 because of the greater yield of both subsets from this cell line.

ELISA reactivity of isolated H-2K^b subsets. The strongly Y3-reactive fractions of peak 1 (pH 8.5 elution) and peak 2 (pH 10.5 elution) isolated from EL4 cells were pooled separately and ELISA reactivity with several K^b-reactive antibodies was determined. From the ELISA results, the mAbs can be grouped into three categories (Table 2-1). The first group includes Y3, 100-30, 5F1 and AF6 which react strongly to both K^b subsets. Y3 is reported to recognize K^b irrespective of association with β_2m or specific peptides (Bluestone et al., 1992; Jackson et al., 1992). The Y3 reactivity by ELISA is high for the pH 8.5- and 10.5-eluted K^b (Table 2-1). In contrast to Y3, the reactivity of 5F1 with K^b is dependent on associated peptide (Bluestone et al., 1992). Reactivity of this mAb is found with both K^b subsets at comparable levels, suggesting that both K^b subsets have bound peptides. Furthermore, both subsets also react with 100-30 and AF6 which recognize peptide-dependent conformational epitopes of K^b (Hogquist et al., 1993).

A second mAb group only includes S19.8. This antibody recognizes K^b associated with the β_2m^b expressed by C57BL/6 and related mouse strains and a bound peptide (Tada et al., 1980; Tatake and Zeff, 1993; Tatake et al., 1995) and reacts exclusively with peak 1, but not the peak 2 subset of K^b and therefore readily discriminates between these two subsets.

A third group includes M1/42 and B8-24-3 which are strongly reactive with peak 2, but react weakly with peak 1. M1/42 mAb is also strongly reactive with peak 2 of ANA-1, RMA, R8 and C1498 cells, but weakly reactive with peak1 (data not shown). Since M1/42 mAb reactivity requires mouse β_2m to be associated with murine class I molecules (Barnabeu et al., 1984), these results demonstrate that peak 2 contains class I molecules with associated murine β_2m. Therefore both isolated subsets contain K^b associated with murine β_2m. The B8-24-3 mAb recognizes a conformational epitope dependent on β_2m and peptide association, providing further evidence that peak 2 contains peptide-MHC complexes. Based on the serological characteristics of the two K^b subsets determined by solid-phase ELISA, we operationally define the pH 8.5-eluted K^b subset as Ly-m11$^+$ and the subsequent pH 10.5-eluted K^b subset as M1/42^{high}.

Allo-specific CTL clones bind and are triggered by the M1/42^{high} but not the Ly-m11$^+$ K^b subset. Due to the serological differences detected in the previous ELISA studies (Table 2-1) between the two K^b subsets, we tested the ability of each isolated subset immobilized on solid-phase to stimulate antigen-specific binding and degranulation by K^b allo-specific CTL clones (Kane et al., 1989b). Substantial binding, 40-60% of the input CTL, was observed to the immobilized M1/42^{high} K^b subset. In contrast, only minimal binding above background, 1-5%, could be detected with the immobilized Ly-m11$^+$ K^b subset (Fig 2-3 A).

When CTL degranulation triggered by immobilized Ly-m11$^+$ and M1/42^{high} K^b subsets was examined, similar results to the CTL binding were obtained (Fig. 2-3 B). We found that the M1/42^{high} K^b subset stimulated degranulation of allo-specific CTL clones as measured by SE release, whereas the Ly-m11$^+$ K^b subset did not (Fig. 2-3 B). The degranulation response of the three CTL clones, elicited by the M1/42^{high} K^b subset, is comparable to that triggered by K^b-bearing EL4 allogeneic target cells (Kane et al., 1989b). Equivalent densities of Y3-reactive K^b of both subsets were used in the binding and SE assays , while the contrasting S19.8 and M1/42 reactivity of each subset was also confirmed by ELISA (Fig. 2-3 C).

Additional allo-specific CTL clones 35 and 13 (Kane et al., 1988 & 1989b), also displayed strong adhesion and response to the M1/42^{high} K^b but no adhesion or response to the Ly-m11$^+$ K^b subset of immunoaffinity purified K^b on solid-phase. Taken together, the results in Figure 2-3 suggest that there are functional differences between the Ly-m11$^+$ and M1/42^{high} K^b subsets; the allo-CTL bind and degranulate in response to the M1/42^{high} K^b subset, but not the Ly-m11$^+$ K^b subset.

Dose-response relationship of CTL recognition of K^b subsets. The Ly-m11$^+$ and M1/42^{high} K^b subsets were serially diluted and immobilized separately on solid-phase and assayed for binding and stimulation of degranulation of allo-specific CTL clone 11. At the highest density shown, both K^b subsets reached equivalent saturating densities as detected by solid-phase Y3 ELISA. The CTL SE release response to the M1/42^{high} K^{b} subset shows a titration curve that includes a threshold density for stimulation and rises to a maximal response at saturating density (Fig. 2-4). Adhesion of the CTL clones to the immobilized M1/42^{high} K^{b} subset closely paralleled the dose-response curve of SE release. In contrast, the Ly-m11^{+} K^{b} subset could not be bound or stimulate the CTL clone at any density, even when the Ly-m11^{+} K^{b} subset is at a saturating and supra-physiological density (Fig. 2-4). Other K^{b} allo-specific CTL clones including clone 35 and AB.1 also were bound to and were stimulated by the M1/42^{high} K^{b} subset, but did not bind or respond to the Ly-m11^{+} K^{b} subset.

The Ly-m11^{+} subset K^{b} molecules do not inhibit CTL recognition of the M1/42^{high} K^{b} subset. It has been reported that an "inhibitory activity", presumably a lipid or lipid-like molecule which inhibited T cell proliferation, could be co-purified with MHC class I antigens (Stallcup et al., 1984). We tested the possibility that the Ly-m11^{+} K^{b} subset contained such inhibitory activities that could prevent or interfere with recognition of K^{b} alloantigen by CTL. As expected, plate wells immobilized with the Ly-m11^{+} K^{b} subset did not stimulate the CTL, whereas the M1/42^{high} K^{b} subset did stimulate significant antigen-specific CTL degranulation (Fig. 2-5). Coimmobilization of the non-stimulatory Ly-m11^{+} K^{b} subset with the M1/42^{high} K^{b} did not inhibit the antigen-specific CTL degranulation response to the M1/42^{high} K^{b} subset (Fig. 2-5). Since the Ly-m11^{+} K^{b} subset does not inhibit CTL recognition of M1/42^{high} K^{b} subset, it is therefore unlikely that inhibitory activities are present within the Ly-m11^{+} K^{b} subset that could readily explain the lack of response to the Ly-m11^{+} K^{b} subset by allo-specific CTL.

The M1/42^{high} but not the Ly-m11$^+$ K^b subset serves as a CTL TCR ligand. The failure of the Ly-m11$^+$ K^b subset to trigger allo-CTL could be due to an inability to engage and trigger TCR and/or CD8 molecules on the CTLs (Kane and Mescher, 1993). It has been demonstrated that a suboptimal density of purified class I alloantigen although insufficient to trigger a full CTL response, it can still activate via TCR engagement, "avidity enhanced" CD8 binding to coimmobilized non-Ag class I molecules, resulting in enhanced CTL binding and degranulation response (Kane and Mescher, 1993). If the Ly-m11$^+$ K^b subset can serve as a TCR ligand, it should trigger enhanced CD8-dependent CTL binding and possibly a degranulation response when coimmobilized with non-Ag class I molecules.

When the Ly-m11$^+$ K^b (possible TCR ligand) was coimmobilized with K^k (non-Ag class I, activated CD8 ligand) we could not detect TCR-triggered CTL binding or degranulation of CTL clone 11 (Fig. 2-6 A and B). At no density could the Ly-m11$^+$ K^b subset trigger clone 11 CTL with or without coimmobilization with non-Ag class I. In contrast, although the M1/42^{high} K^b subset at a suboptimal density was not sufficient to trigger the allo-CTL clone 11 to bind and degranulate alone, it triggered clone 11 to bind to coimmobilized K^k and degranulate, indicating that the M1/42^{high} K^b subset serves as a TCR ligand (Fig. 2-6 A and B). The TCR-triggered binding or degranulation of the CD8$^+$ CTL clone was not observed when either K^b subset was coimmobilized with class II MHC control molecules (Fig. 2-6 A and B). These results suggest that the immunoaffinity purified M1/42^{high} K^b subset serves as a TCR ligand, upregulating CD8 adhesion and signalling function, but the Ly-m11$^+$ K^b subset does not serve this function (Fig. 2-6).

Both the Ly-m11$^+$ and M1/42^{high} K^b subsets are activated CD8 ligands. Since the preceding results showed that only the M1/42^{high} K^b subset could serve as a TCR ligand, we sought to determine whether either subset could serve as an activated CD8 ligand. It has been shown that soluble anti-TCR antibody activates adhesion of CD8$^+$ CTL to class I, but not class II MHC proteins (O'Rourke et al., 1990). The adhesion is mediated by CD8, since it is effectively inhibited by anti-CD8 antibody (O'Rourke et al., 1990). When the D^d allo-specific CTL clone 10/1 was treated with soluble anti-CD3 antibody, 145-2C11, it bound and degranulated in response to immobilized non-Ag class I (K^k), but not class II (I-A^d) proteins (Fig. 2-7 A and B). A substantial and similar degree of TCR-activated CTL binding was observed for both immobilized Ly-m11$^+$ and M1/42^{high} K^b subsets (Fig. 2-7 A), and CTL binding to both K^b subsets could be effectively blocked by anti-CD8 mAb (Fig. 2-7 A inset). In addition, strong degranulation responses to immobilized K^b subsets were observed, with the Ly-m11$^+$ subset providing somewhat stronger CD8 costimulation than the M1/42^{high} K^b subset (Fig. 2-7 B). From these observations, we concluded that both K^b subsets can serve as avidity-enhanced CD8 ligands, and the Ly-m11$^+$ K^b subset may possibly deliver more efficient signals through CD8 to activate CTL degranulation.

The lack of TCR recognition of Ly-m11$^+$ K^b subset is not due to the absence of relevant bound endogenous peptides. That most allo-specific CTL recognize an allo-class I molecule bound with specific endogenously processed peptide(s) (Heath et al., 1991; Rotzschke et al., 1991; Sherman and Chattopadhyay, 1993) suggested the possibility that the functional differences exhibited by the Ly-m11$^+$ and M1/42^{high} K^b subsets could reflect differences in the composition of endogenous peptides bound by the two K^b subsets. To test this possibility, we acid-eluted endogenous peptides from equivalent amounts of the two isolated K^b subsets, based on their Y3 ELISA reactivity, and assayed them for the ability to sensitize T2K^b cells for lysis by K^b-specific allo-CTL (Heath et al., 1991; Chattopadhyay et al., 1994). The T2K^b cells are believed to express peptide "empty" K^b molecules and they are efficient at binding and presenting exogenous peptide antigens (Wei and Cresswell, 1992). Although allorecognition can be peptide-independent in some cases (Smith et al., 1997), lysis of T2K^b cells by allo-specific CTL clone 35 and clone 11 is dependent on endogenous peptides eluted from isolated K^b (Fig. 2-8 A and B). This is consistent with the previous report that lysis of K^b expressing targets by both K^b allo-specific CTL clone 35 and 11 are dependent on unidentified short endogenous peptides generated in EL4 cells (Heath et al., 1989).

Peptides eluted from both Ly-m11$^+$ and M1/42^{high} K^b subsets sensitized T2K^b for lysis to comparable levels, by clone 35 (Fig. 2-8 A) and clone 11 (Fig. 2-8 B). Titration of the eluted peptides from the K^b subsets for the T2K^b sensitization indicates that the yields of the relevant peptides mediating allorecognition are similar for the Ly-m11$^+$ and M1/42^{high} K^b subsets (Fig. 2-8 B). From these results, we conclude that the relevant peptides for recognition by the allo-specific CTL clones are associated with both the Ly-m11$^+$ and M1/42^{high} K^b subsets. Furthermore, the appropriate peptides do not appear to be limiting in the Ly-m11$^+$ subset, hence it is unlikely that associated peptides provide an explanation for the differences in functional activities of the Ly-m11$^+$ and M1/42^{high} K^b subsets.

The Ly-m11$^+$ and M1/42^{high} K^b subsets differ in their N-linked glycosylation. We attempted to determine the biochemical basis for the observed serological and functional differences between the two K^b subsets. The

SDS-PAGE analysis with Coomassie blue staining revealed a 47 kD K^b heavy chain (Fig. 2-9 *a*, lane 2, solid arrow) and a 12 kD β2m (not shown) in the M1/42^{high} K^b subset. Two major bands were seen in the Ly-m11$^+$ K^b subset; one band at 51 kDa (Fig. 2-9 *a*, lane 1, barbed arrow) with a barely visible smear on top and another band at 43 kD (Fig. 2-9 *a*, lane 1, open arrow). The identity of the 43 kD band is unclear, however it is Endo H and Endo F insensitive (data not shown) and is therefore likely to be a contaminant such as actin, which has been seen in class I immunoprecipitations (Jones, 1977; Gilmer, 1982; Williams et al., 1985 & 1988). For the Ly-m11$^+$ K^b subset, the β_2m is less readily seen in Coomassie staining, but is visualized by silver staining.

When the K^b subsets were purified from EL4 cells labelled with ^{125}I and visualized by autoradiography, a clear difference in M_r between the two K^b subsets was again observed. The M1/42^{high} K^b is a 47 kD band (Fig. 2-9 *b*, lane 4); in contrast, the Ly-m11$^+$ K^b subset was visualized as a broad 51-56 kD band likely to be made up of multiple closely migrating bands (Fig. 2-9 *b*, lane 1). This 51-56 kD broad band most probably includes the 51 kD band on the Coomassie blue staining gel in Figure 2-9 *a*. Since both EL4 K^b subsets were labelled by ^{125}I, we presume that they are both cell surface forms of K^b. Higher M_r H-2K^b molecules with a similar migration and banding pattern to that observed in the EL4 Ly-m11$^+$ K^b subset have been identified in immunoaffinity purified preparations of K^b from the B/6 murine leukemia cell line RBL-5 and may represent similar K^b species (Rogers et al., 1979; Rogers, 1981).

Since the apparent differences in migration of the Ly-m11$^+$ and M1/42^{high} K^b subsets on SDS-PAGE could be due to differences in glycosylation, we performed Endo H, Endo F and NANAse treatment of both K^b subsets. After Endo F treatment, both the 51 - 56 kD broad band in the Ly-m11$^+$ K^b subset (Fig. 2-9 *b*, lane 1) and the 47 kD band in the M1/42^{high} K^b subset (Fig. 2-9 *b*, lane 4) shifted in mobility to a position corresponding to 40 kD (Fig. 2-9 *b*, lane 3 and 6), which is consistent with the previous report (Landolfi et al., 1985). This result suggests that: 1) both K^b subsets are *N*-linked glycosylated, and 2) their peptide backbones are of the same or very similar size, making it very unlikely that the difference in M_r of the two K^b subsets is due to differential splicing (Robinson and Kindt, 1989). The fact that differentially spliced forms of K^b were not found on the surface of EL4 cells (Lew et al., 1986) also argues against the possibility that two subsets of K^b are products of mRNA differential splicing. Both subsets were found to be resistant to Endo H treatment (Fig. 2-9 *b*, lane 2 and 5), indicating that the *N*-linked carbohydrates on both subsets are of a mature or complex form. Upon NANAse treatment, the 51-56 kD band of the Ly-m11$^+$ K^b subset shifted only very slightly towards the gel bottom (Fig. 2-9 *c*, lane 2). This suggests either that sialylation is not the major factor responsible for the mobility difference between the Ly-m11$^+$ and M1/42^{high} K^b subsets, or there exist sialic acid-containing carbohydrates on the Ly-m11$^+$ K^b subset that are resistant to NANAse treatment (Krakauer et al., 1980). Occasionally, upon longer exposure, we see a faint 47 kD band which is corresponding to the size of the M1/42^{high} K^b band in the Ly-m11$^+$ K^b subset (Fig. 2-9 *c*) which may or may not be Ly-m11 reactive or possibly represents some contaminating M1/42-reactive K^b. In summary, the Ly-m11$^+$ and M1/42^{high} K^b subsets differ in their *N*-linked glycosylation in addition to their serological and functional differences. The observed variance in glycosylation between the two K^b subsets may be responsible for their observed functional differences.

D. Summary of Results

In the present study, two cell surface subsets of murine H-2 K^b were isolated from detergent lysates of EL4 and other H-2K^b bearing cells. These K^b subsets were found to differ serologically; although both subsets share similar Y3 reactivity, one subset is Ly-m11$^+$ and the other is predominantly reactive with the M1/42 antibody. The two K^b subsets differed dramatically in their abilities to serve as TCR ligands for allo-specific CTL adhesion, and transmembrane signalling as measured by alloantigen-specific degranulation. The two K^b subsets also varied significantly in their *N*-linked glycosylation, and this may relate to the functional differences observed between the two K^b subsets.

E. Discussion

Both subsets of K^b are heavy chain/β2m/peptide complexes and contain relevant peptides for allo-CTL recognition. We have demonstrated herein that two immunoaffinity purified K^b subsets differ in their serology, glycosylation and ability to trigger allo-specific CTL. The serological characteristics of the two K^b subsets (Table 2-1) indicate that each contains K^b heavy chain associated with β_2m as the epitopes recognized by S19.8 (Tatake and Zeff, 1993; Tatake et al., 1995), M1/42 (Bernabeu et al., 1984) and AF6 (Bluestone et al., 1992) mAbs are dependent on class I heavy chain association with murine β_2m. Furthermore, since both K^b subsets are reactive with several mAbs that are dependent on peptide association with K^b (Bluestone et al., 1992; Jackson et al., 1992; Hogquist et al., 1993), both subsets contain endogenous peptides. Complexes of class I MHC molecules with different endogenous peptides can be distinguished from each other by allo-antibodies (Bluestone et al., 1992; Catipovic et al., 1992;

Jackson et al., 1992; Hogquist et al., 1993; Sherman et al., 1993). These serologically defined differences may be due, in part, to subtle peptide-dependent conformational variations in the class I heavy chain (Bluestone et al., 1992; Catipovic et al., 1992), or to differences in the size of exposed peptide side chains that may affect antibody binding (Hogquist et al., 1993). The serological differences between the two K^b subsets could suggest that certain endogenous peptides may be predominantly bound to one or another K^b subset (Noun et al., 1996). Despite the observed serological differences, relevant peptides could be extracted from both K^b subsets with comparable yield for sensitizing $T2K^b$ cells for alloantigen-specific CTL lysis. These results indicate that the functional differences between the two K^b subsets are not likely to be due to the absence or insufficiency of the relevant peptide(s) bound with K^b.

The Ly-m11⁺ K^b is not inhibitory to CTL responses. Given that both K^b subsets contain sufficient amounts of relevant peptides for allo-CTL stimulation, another possibility for why the Ly-m11$^+$ K^b subset did not trigger the CTL clones; namely the Ly-m11$^+$ K^b subset contains "inhibitory" or antagonistic activity that prevents CTL from functioning, is considered. Firstly, it was reported that affinity purified K^k preperations contained lipids or lipid-like substances that could inhibit lymphoid cell proliferation (Stallcup et al., 1984). Similar substances could be present in the Ly-m11$^+$ K^b subset. Secondly, the Ly-m11$^+$ K^b subset contains peptide-K^b complexes that are TCR antagonists, similar to what has been reported for complexes of MHC molecules and certain synthetic peptide antigen analogs (Evavold et al., 1993; Jameson et al., 1993). However, our results indicate that the Ly-m11$^+$ K^b subset is unable to inhibit the binding and response of the allo-specific CTL clones when coimmobilized with stimulatory M1/42^{high}

K^b subset molecules, suggesting that the Ly-m11$^+$ K^b subset does not apparently contain strongly inhibitory or antagonistic activities.

Differential glycosylation of MHC class I. We observed a significant difference in M_r of the two K^b subsets, with the M1/42^{high} K^b subset being approximately 47 kD and the Ly-m11$^+$ K^b subset ranging from 51-56 kD. Murine class I at the higher M_r has been occasionally observed before in immunoprecipitates (Le and Doyle, 1982; Thor et al., 1993). When present in immunoprecipitates, the higher M_r class I is sometimes assumed to represent class I heavy chain with β_2m still associated. However, we consider this possibility for the isolated Ly-m11$^+$ K^b subset very unlikely since there was no change in the migration of this subset in gels run in the presence of 8 M urea and 100 mM dithiothreitol. Since the Ly-m11$^+$ K^b subset migrates on SDS-PAGE in a diffuse pattern and is more acidic than the M1/42^{high} K^b by isoelectric focusing, these results more likely suggest that differences in glycosylation may be contributing to the observed heterogeneity between the two isolated K^b subsets. Consistent with this possibility, we found that upon Endo F treatment to remove *N*-linked carbohydrate structures, both K^b subsets migrated coincidently. Murine class I molecules have 2 or 3 *N*-linked glycans with K^b usually possessing two *N*-linked complex-type glycosylations at Asn-86 and Asn-176 of the α_1 and α_2 domains, respectively (Klein, 1986). Microheterogeneity of murine class I molecules has been observed previously and has been attributed to differences in the carbohydrate structures attached to the heavy chain (Jones, 1977; Le and Doyle, 1982; Swiedler et al., 1985). Often two major subsets that differ in size as well as charge can be observed by two-dimensional gels from immunoprecipitations of some H-2 class I molecules (Jones, 1977; Gilmer, 1982; Le and Doyle, 1982). In some cases the decreased mobility and higher negative charge of one subset can be attributed at least in part to greater sialylation (Parham et al., 1974; Jones, 1977; Krakauer et al., 1980; Le and Doyle, 1982; Swiedler et al., 1985).

Despite the fact that class I carbohydrate microheterogeneity has been shown to exist, we demonstrate in this report that at least two subsets of cell surface expressed K^b, differing in *N*-linked carbohydrate modification can be independently isolated and analyzed biochemically and functionally. The higher M_r of the Ly-m11$^+$ K^b may possibly be due to differences in its degree of sialylation relative to the M1/42^{high} K^b subset. However, the relative resistance of the Ly-m11$^+$ K^b to NANAse treatment suggests either that sialylation is not the major contributor to the reduced mobility or that sialylation may be responsible, but most of the sialic acid linkages are of a type that is resistant to routine NANAse treatment (Krakauer et al., 1980). An alternative or additional possibility is that the hyperglycosylated Ly-m11$^+$ K^b may be modified by poly-*N*-acetyllactosamine (PL) addition(s) similar to ABO blood group antigens that can be found on RBC and hematopoietic cells (Fukuda, 1985). Multiple and variable lactosaminyl unit additions can result in a mixture of multiple sizes of a glycoprotein (Fukuda, 1985), observed as broadening and smearing in SDS-PAGE, similar to what we observe for the Ly-m11$^+$ K^b subset.

Differential glycosylation of MHC class I affects mAb recognition. It has been observed previously that the *N*-linked carbohydrate on murine class I molecules may affect their recognition by some monoclonal antibodies (Shiroshi et al., 1985; Miyasaki et al., 1986; O'Neill, 1991). It is presently unclear why the Ly-m11$^+$ K^b subset is more readily eluted from the Y3 column than the M1/42^{high} K^b subset. However, it is conceivable that the affinity of the Y3 mAb for the Ly-m11$^+$ subset may be diminished due to the apparent greater size of the carbohydrate side chains on the Ly-m11$^+$ K^b subset. It is possible for the same reason, that the larger carbohydrate found on the Ly-m11$^+$ K^b subset negatively affects M1/42 mAb recognition possibly due to steric hindrance, masking of an epitope or it lacks a specific carbohydrate structure that may be involved in M1/42 recognition of class I. Recognition of K^b complexes by the S19.8 (Ly-m11) mAb may be enhanced by certain carbohydrate structures found on one, but not the other K^b (M1/42^{high}) subset. Further studies will be required to determine the influence of *N*-linked carbohydrate structure on M1/42 and S19.8 antibody recognition of K^b molecules.

Effects of excessive glycosylation of MHC class I on allo-CTL recognition. It has been reported previously that *N*-linked carbohydrates of both murine and human MHC class I are not required for CTL recognition (Goldstein and Mescher, 1985; Miyasaki et al., 1986; Barbosa et al., 1987). Removal of *N*-linked carbohydrate from K^k by Endo F treatment does not affect allo-specific CTL proliferation (Goldstein and Mescher, 1985), and amino acid substitutions at all three Asn carbohydrate attachment sites on L^d by site-directed mutagenesis also did not result in a significant change in allo-CTL lysis of cells transfected with the mutated L^d gene (Miyasaki et al., 1986). Despite observations indicating that the *N*-linked carbohydrates on class I seem not to be required for CTL recognition, the effects of the extent and composition of the class I carbohydrate side chains on CD8$^+$ CTL recognition is not understood. Degen et al. (1989) found that the murine tumor cell line, MDAY-D2, did not elicit strong CTL response as its WGA-resistant variant, MDW3, did. They compared and correlated their H-2 class I (D^d) molecule carbohydrate modifications and their lysis by D^d-restricted anti-tumor CTL. The oligosacchrides isolated from MDAY-D2 D^d seemed to be slightly larger and more branched as compared to its variant, MDW3. However, when both MDAY-D2 and MDW3 cells were treated with the oligosacchride processing inhibitor, swainsonine, to convert their surface class I oligosacchrides to simpler hybrid structures, lysis of both tumor cells by anti-MDW3 CTL was not affected. Therefore, it was concluded that large carbohydrate structures on class I themselves were not responsible for their poor lysis. It is possible that the "larger" oligosacchride structure is not large enough to be the major contributor of the difference of recognition between the two tumor cells. Or, the carbohydrate structures lack the components that are critical for interfering with CTL recognition. Additionally and more importantly, there was no direct functional comparison between the differentially glycosylated D^d molecules.

In the present study, we have independently isolated class I molecules with significantly different carbohydrate structures, and analyzed them biochemically and functionally. Our data suggest for the first time that the type of *N*-linked carbohydrate on class I is likely to affect TCR recognition of class I alloantigen. How might the carbohydrate expressed on the Ly-m11$^+$ K^b subset negatively affect TCR recognition? Several explanations are possible including: 1) because of their larger size, the carbohydrate structures on the Ly-m11$^+$ K^b subset may sterically block TCR interaction(s); 2) the Ly-m11$^+$ K^b subset expresses carbohydrate with a high net negative charge due to a high degree of NANAse resistant sialylation or possibly sulfation, resulting in repulsion between the TCR and class I; 3) the carbohydrate on the two K^b subsets may affect the conformation of the heavy chain differentially; subtle changes in class I conformation are known to affect TCR recognition (Bluestone et al., 1993; Chattopadhyay et al., 1994); 4) some combination of the above possibilities. That the too abundant carbohydrate structures potentially block TCR contact with class I molecules is particularly appealing, since both the K^b *N*-linked glycosylation sites (Asn86 in the $\alpha 1$ and Asn 176 in $\alpha 2$ domain) are located extremely close to the TCR contact sites (Parham, 1996 a & b), and it is likely that bulky oligosaccharide structure(s) could interfere with TCR interaction.

We have treated the Ly-m11$^+$ K^b subset with Endo F or NANAse to remove *N*-linked glycosylation or its terminal sialic acids respectively, in order to convert the Ly-m11$^+$ K^b subset to a functional TCR ligand. Although K^k has been shown to be stable and functional after exhaustive enzyme digestions (Goldstein and Mescher, 1985), however, with similar methods, the K^b in both subsets is unfortunately not sufficiently stable following enzyme treatments to assay for CTL recognition.

The Ly-m11 epitope has been detected on the surface of a variety of normal and transformed cell types (Tada et al., 1980; Tatake and Zeff, 1993). Almost all spleen and lymph node cells and a portion of bone marrow and thymus cells express this K^b and β2m-dependent epitope. Tumors cells derived from various hematopoietic cell types from C57BL/6 and related strains are also positive for this antigen. Consistent with these previous observations, we were able to purify Ly-m11$^+$ K^b from a variety of H-2^b expressing tumor cell types of hematopoietic origin. We have also attempted to purify the two K^b subsets from normal spleen and thymus cells, however the yields of K^b were not sufficient to be tested in assays done with both K^b subsets isolated from tumor cells. Therefore, a direct comparision of K^b subsets from normal and transformed cells has not been possible and their potential structural and functional relationships remain unresolved.

Table 2-1. ELISA reactivity of H-2 K^b subsets isolated from EL4 cells[1]

mAb[2]	Peak 1 (pH8.5 elution)	Peak 2 (pH10.5 elution)
Y3	0.861 ± 0.051[3]	0.885 ± 0.038
5F1	0.361 ± 0.028	0.488 ± 0.024
100-30	0.590 ± 0.050	0.842 ± 0.071
AF6	0.662 ± 0.056	0.722 ± 0.091
S19.8 (Ly-m11)	0.852 ± 0.054	0.005 ± 0.012
M1/42	0.033 ± 0.016	0.917 ± 0.026
B8-24-3	0.153 ± 0.023	0.812 ± 0.083

[1] ELISA reactivity of isolated EL4 K^b subsets to various mAbs was determined. From a representative preparation, ELISA peak fractions of pH 8.5- and pH 10.5-eluted K^b were pooled separately, diluted 1:20 into D-PBS was for all assays.

[2] First antibody used in ELISA assay: Y3 (2.1 mg/ml) and M1/42 (1.5 mg/ml) were purified and diluted a 1:500 in 2% FCS-PBS; 5F1 was used as ascites at a 1:100 dilution; 100-30, AF6, B8-24-3 and S19.8 (Ly-m11) were used as culture supernatants in 5% FCS-RPMI at 1:4 dilution.

[3] Results are expressed as mean OD_{490nm} ± SD of triplicate wells.

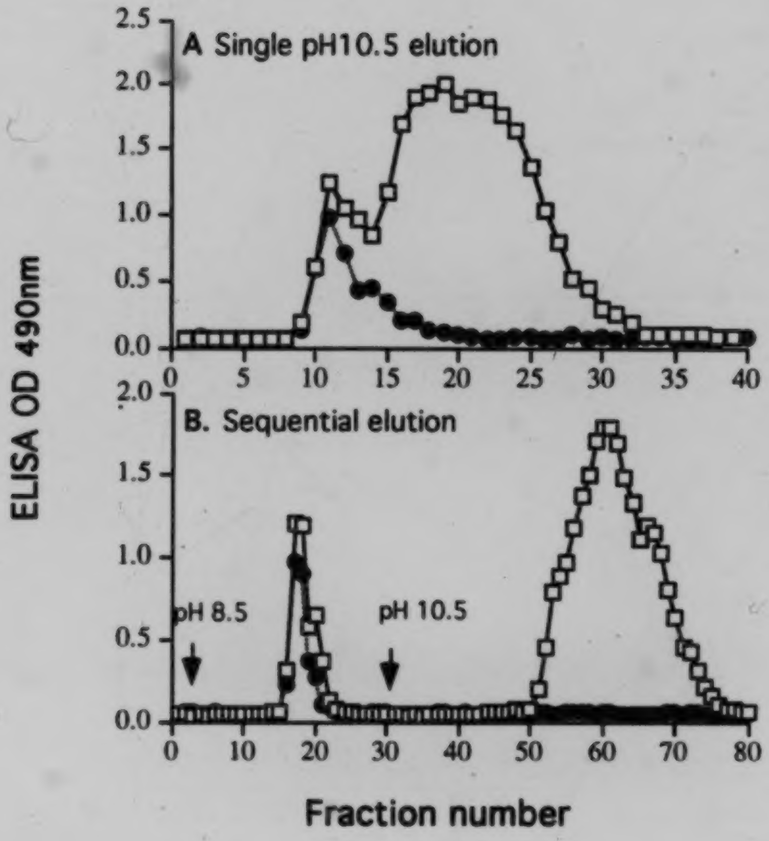


Figure 2-1. Isolation of two serologically distinct K^b subsets from EL4 cells. K^b molecules were isolated from EL4 cells by Y3 mAb immunoaffinity chromatography. Elutions of K^b from Y3 mAb column were performed by (A) a single pH 10.5 elution or (B) sequential application of pH 8.5 and pH10.5 buffers. The start of pH 8.5 and pH 10.5 elutions is indicated by arrows. Each fraction (1:20 dilution) was assayed for Y3 (□) and S19.8 (Ly-m11) (●) mAb reactivity by solid-phase ELISA. Purified mAb Y3 (2.1 mg/ml) was diluted 1:500, and S19.8 culture supernatant was at a 1:4 dilution. Results are expressed as an average of OD_{490nm} of duplicate wells.

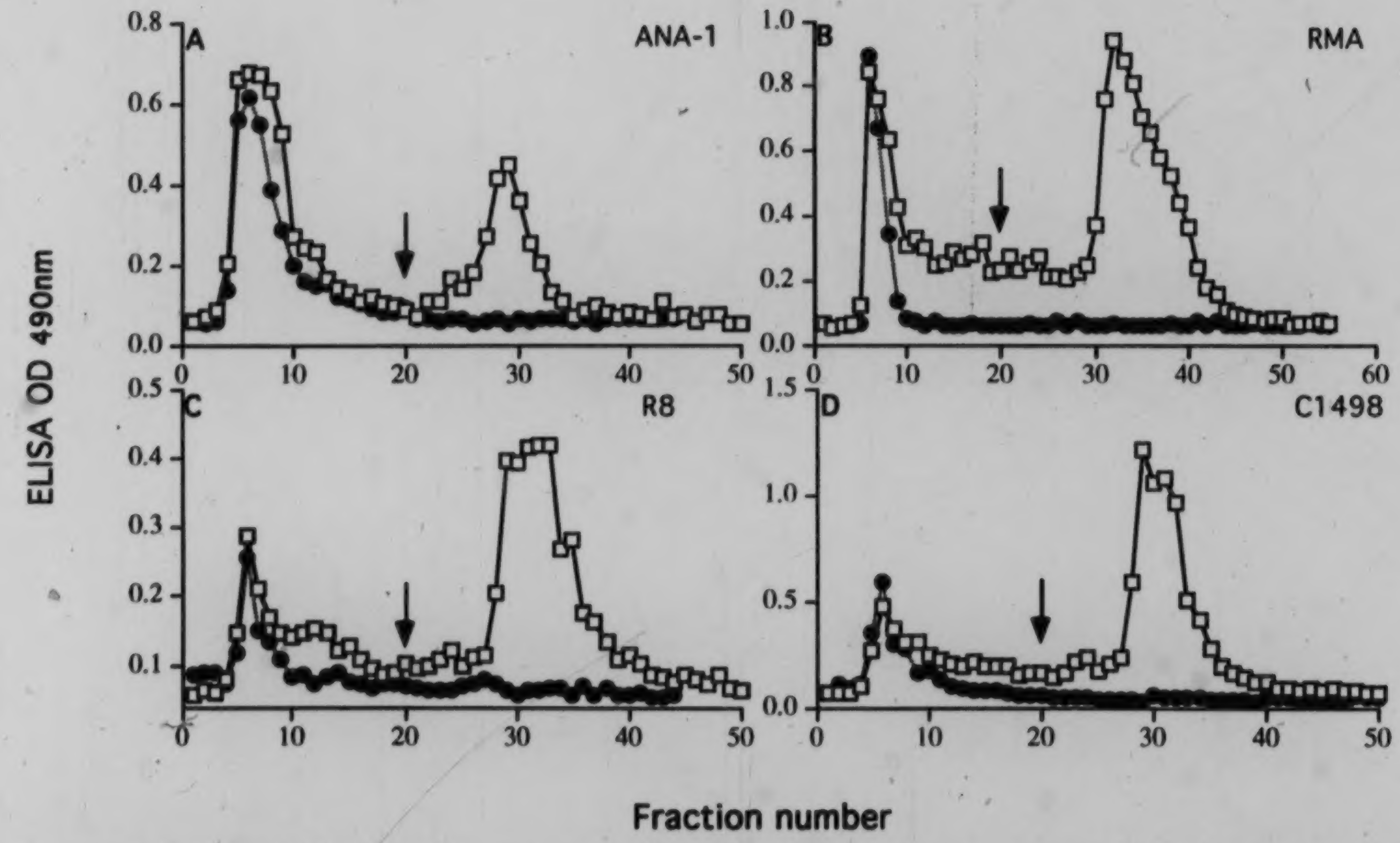
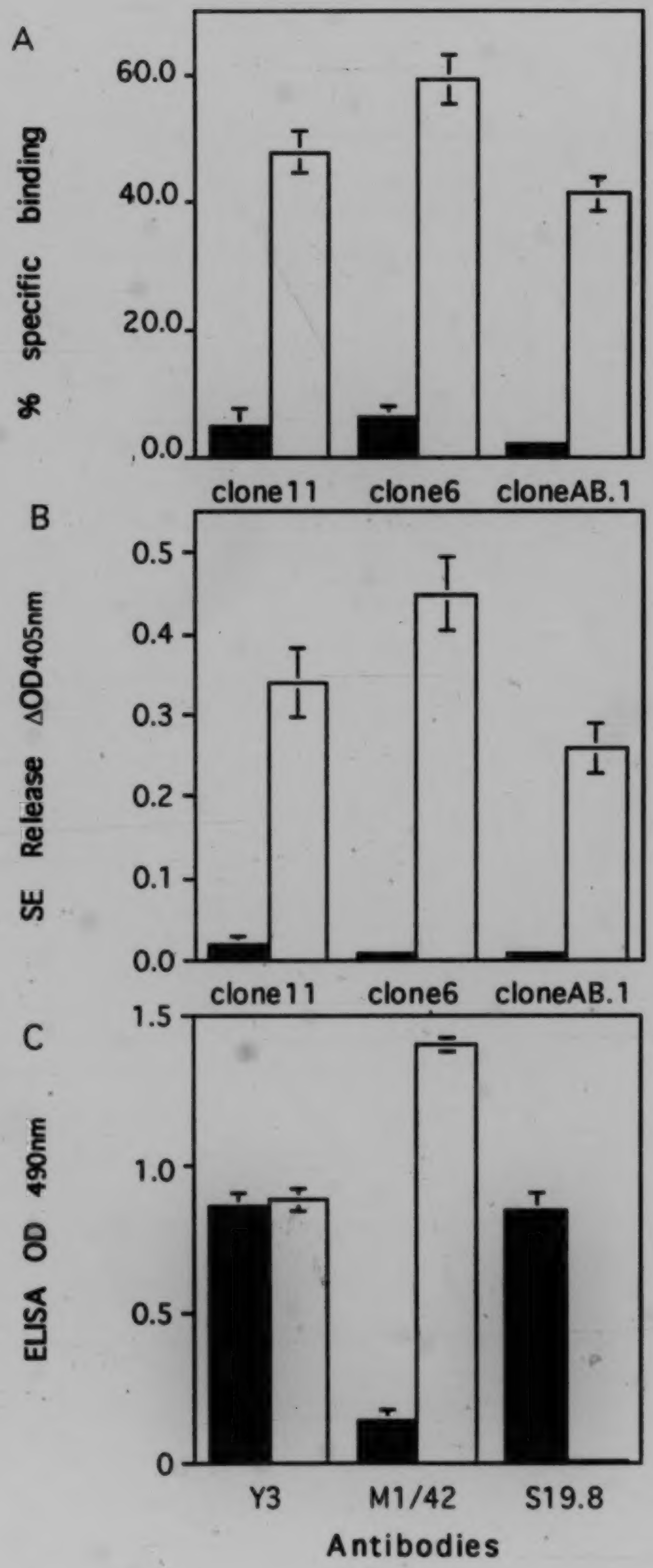


Figure 2-2. Isolation of K^b subsets from various H-2 K^b-expressing cells. K^b molecules were isolated from (A) ANA-1, (B) RMA, (C) R8 and (D) C1498 cells by Y3 mAb immunoaffinity chromatography. Elutions of K^b from Y3 mAb column were performed by sequential application of pH 8.5 and pH10.5 buffers. Arrows indicate the start of pH 10.5 elutions. Each fraction was assayed for Y3 (□) and S19.8 (Ly-m11) (●) mAb reactivity by solid-phase ELISA as described in Figure 2-1.

Figure 2-3. Allo-specific CTL clones bind and are triggered by the M1/42^{high}, but not Ly-m11^{+} K^{b} subset. Antigen-specific binding (A) and SE release (B) of three K^{b} allo-specific CTL clones in response to immobilized Ly-m11^{+} (*solid bar*) and M1/42^{high} (*open bar*) K^{b} subsets were determined. 2 x 10^{5} of each CTL clone were added to each well and incubated for 4 h. Results are expressed as mean ± SD. The same Ly-m11^{+} (*solid bar*) and M1/42^{high} (*open bar*) K^{b} subsets were assayed for their immunoreactivity with Y3, M1/42 and S19.8 mAbs in parallel by ELISA (C) at a density identical to that used in the above CTL binding and SE release experiments. Results are expressed as mean OD$_{490nm}$ ± SD.

Figure 2-3


A
% specific binding
60.0
40.0
20.0
0.0
clone11
clone6
cloneAB.1
B
SE Release ΔOD405nm
0.5
0.4
0.3
0.2
0.1
0.0
clone11
clone6
cloneAB.1
C
ELISA OD 490nm
1.5
1.0
0.5
0
Y3
M1/42
S19.8
Antibodies

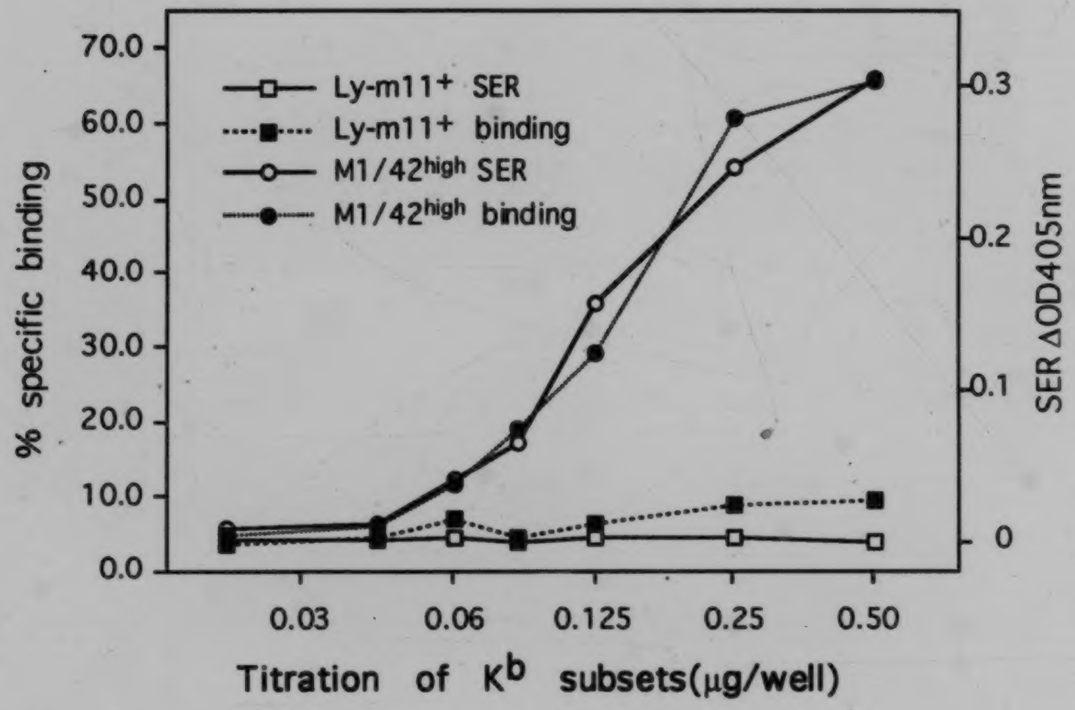


Figure 2-4. Dose-response relationship of CTL recognition of K^b subsets. Both K^b subsets were serially diluted separately and immobilized on plastic. 10^5 clone 11 CTL were added and incubated for 4 h. Results of both CTL binding and SE release were averages of duplicate wells. The variation was less than 10% between wells.

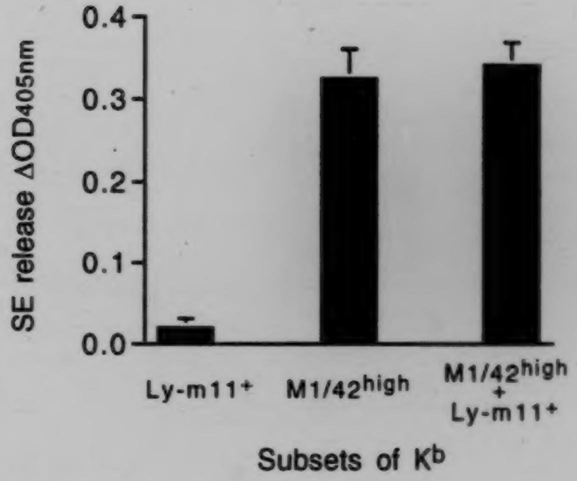


Figure 2-5. The Ly-m11$^+$ K^b subset does not inhibit recognition of the M1/42^{high} K^b subset by an allo-specific CTL clone. Two K^b subsets containing an equivalent amount of Y3-reactive K^b were diluted 1:20 separately in D-PBS and immobilized individually on the plate. For the mixing experiment, both K^b subsets were diluted together into D-PBS in which each K^b subset is 1:20 diluted, and coated on the plate. Clone 11 SE release triggered by Ly-m11$^+$, M1/42^{high}, or the mixture of both K^b subsets was determined.

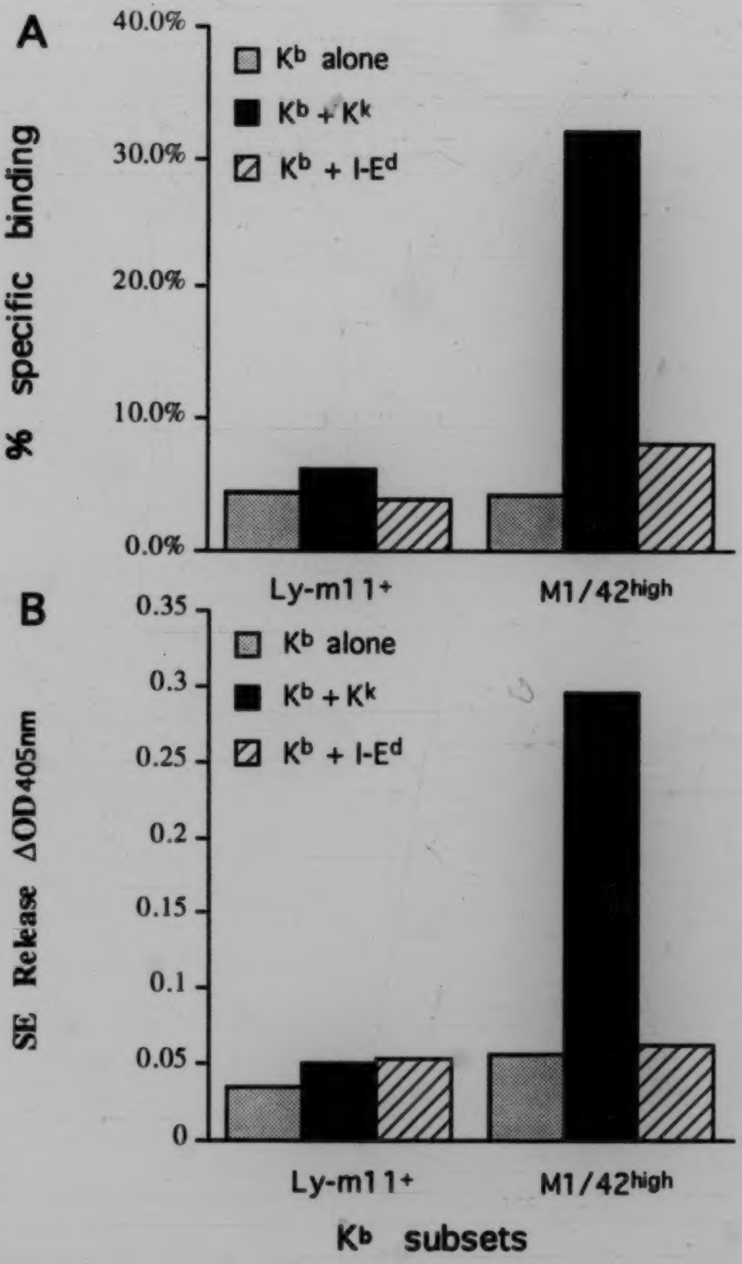
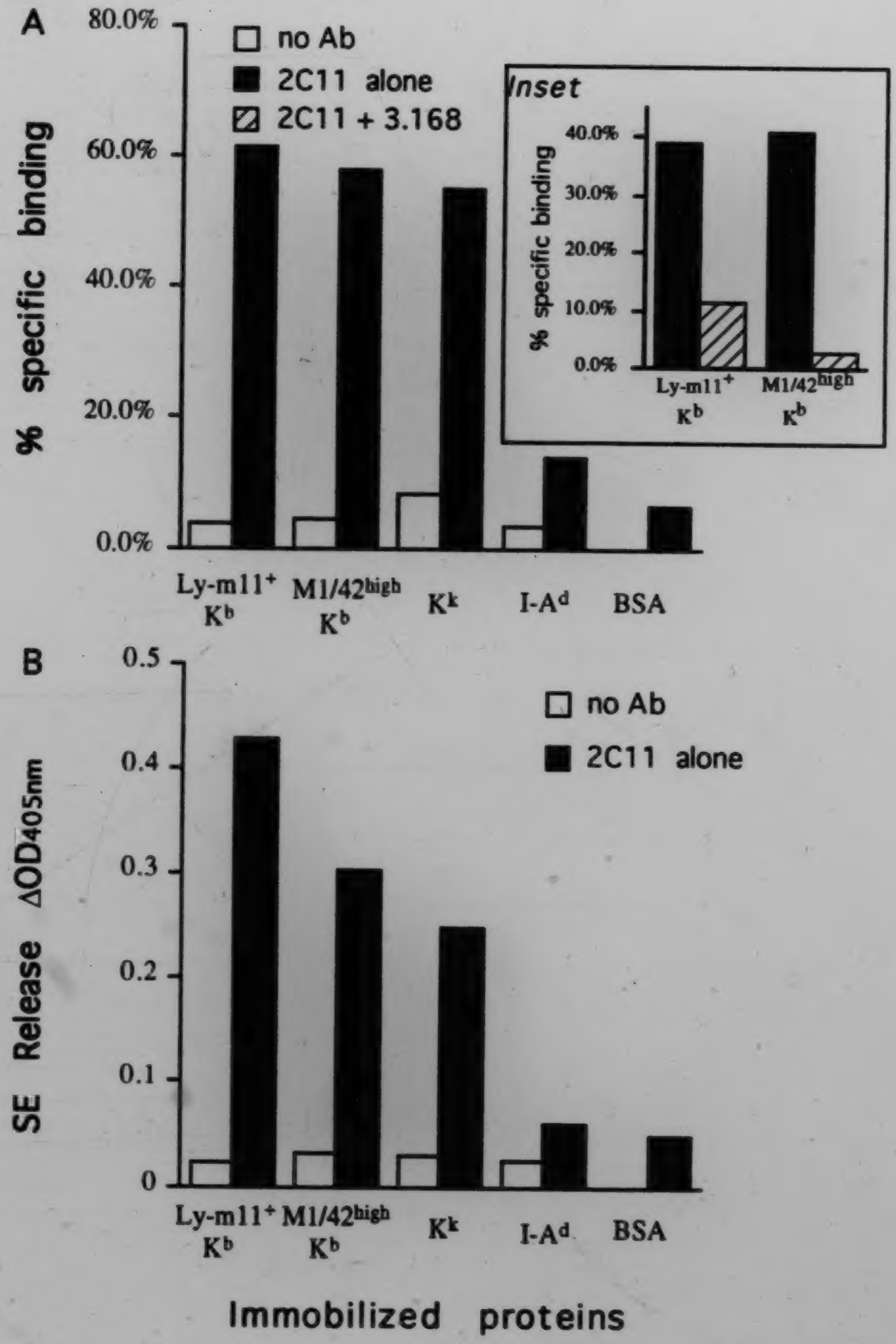


Figure 2-6. The M1/42^{high}, but not the Ly-m11^{+} K^{b} subset, can serve as a TCR ligand. Both the Ly-m11^{+} and M1/42^{high} K^{b} subsets (0.07 µg/well) were separately immobilized alone, or coimmobilized with H-2 K^{k} (0.15 µg/well) or I-E^{d} (0.21 µg/well) as indicated. The wells were blocked with 2% FCS in D-PBS. Clone 11 CTL were added to wells at 1.5 X 10^{5} cells/well, incubated for 4 h at 37°C, and binding (A) and SE release (B) determined. Data were expressed as the mean of triplicate wells.

Figure 2-7. Both the Ly-m11^{+} and M1/42^{high} K^{b} subsets can serve as activated CD8 ligands. The Ly-m11^{+} and M1/42^{high} K^{b} subsets (both at a density of 0.15 μg/well), H-2 K^{k} (0.16 μg/well) I-A^{d} (0.20 μg/well) or BSA (100 μg/well) were separately immobilized on the wells as indicated, and blocked with 2% FCS in D-PBS. The D^{d} allo-specific CTL clone, 10/1, was loaded into the wells at 1.0 X 10^{5} cells/well. Immediately before loading, clone 10/1 cells were treated with 0.25 μg/ml 2C11 mAb (*solid bar*), or without antibody treatment (*open bar*). CTL clone 10/1 binding (A) and SE release (B) were determined. In a separate experiment, the effect of anti-CD8 mAb on clone 10/1 binding was tested (A Inset). Clone 10/1 were incubated at room temperature with 3.168 anti-CD8 mAb at 1:100 dilution of ascites for 30 min before the addition of 2C11 mAb. Clone 10/1 binding in the absence of 2C11 was 3.5%. In the presence of 2C11 mAb, clone 10/1 adhesion with (*hatched bar*) or without (*solid bar*) 3.168 mAb treatment was determined. All data were expressed as the mean of triplicate wells. Binding and SE release of clone 10/1 in the absence of mAb 2C11 in the BSA-coated wells were determined as background and subtracted from the experimental groups.

Figure 2-7


A
80.0%
60.0%
40.0%
20.0%
0.0%
% specific binding
☐ no Ab
■ 2C11 alone
▨ 2C11 + 3.168
Inset
40.0%
30.0%
20.0%
10.0%
0.0%
% specific binding
Ly-m11⁺ Kᵇ
M1/42ʰⁱᵍʰ Kᵇ
Ly-m11⁺ Kᵇ
M1/42ʰⁱᵍʰ Kᵇ
Kᵏ
I-Aᵈ
BSA
B
0.5
0.4
0.3
0.2
0.1
0
SE Release ΔOD405nm
☐ no Ab
■ 2C11 alone
Ly-m11⁺ Kᵇ
M1/42ʰⁱᵍʰ Kᵇ
Kᵏ
I-Aᵈ
BSA
Immobilized proteins

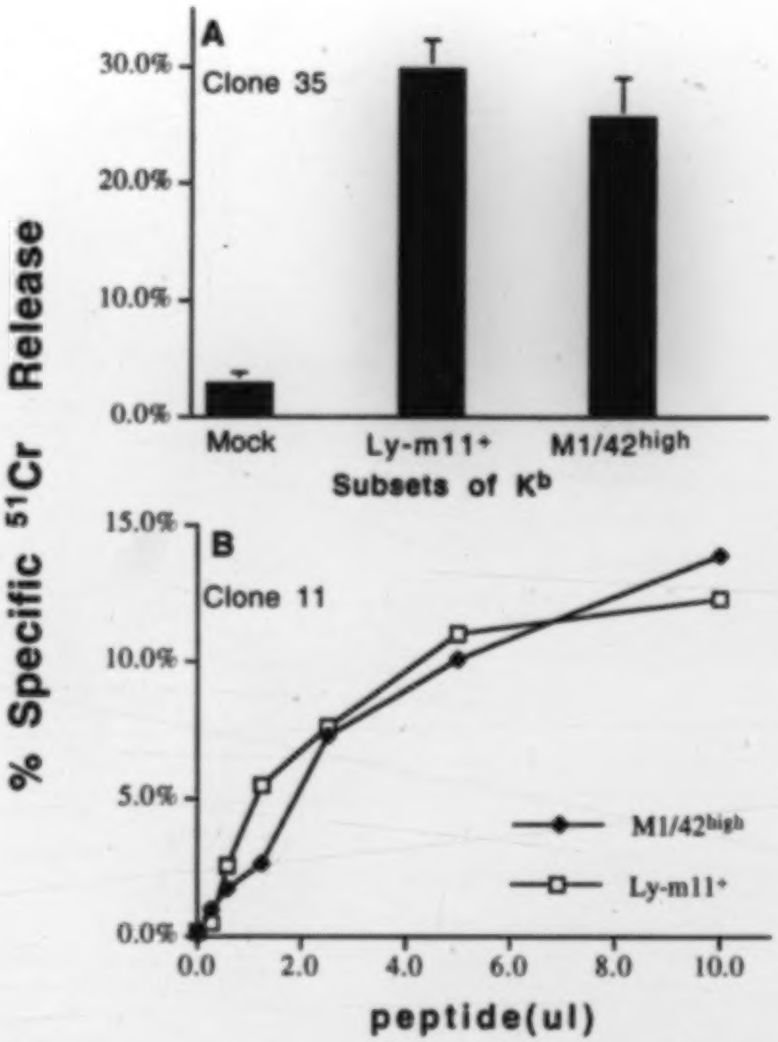
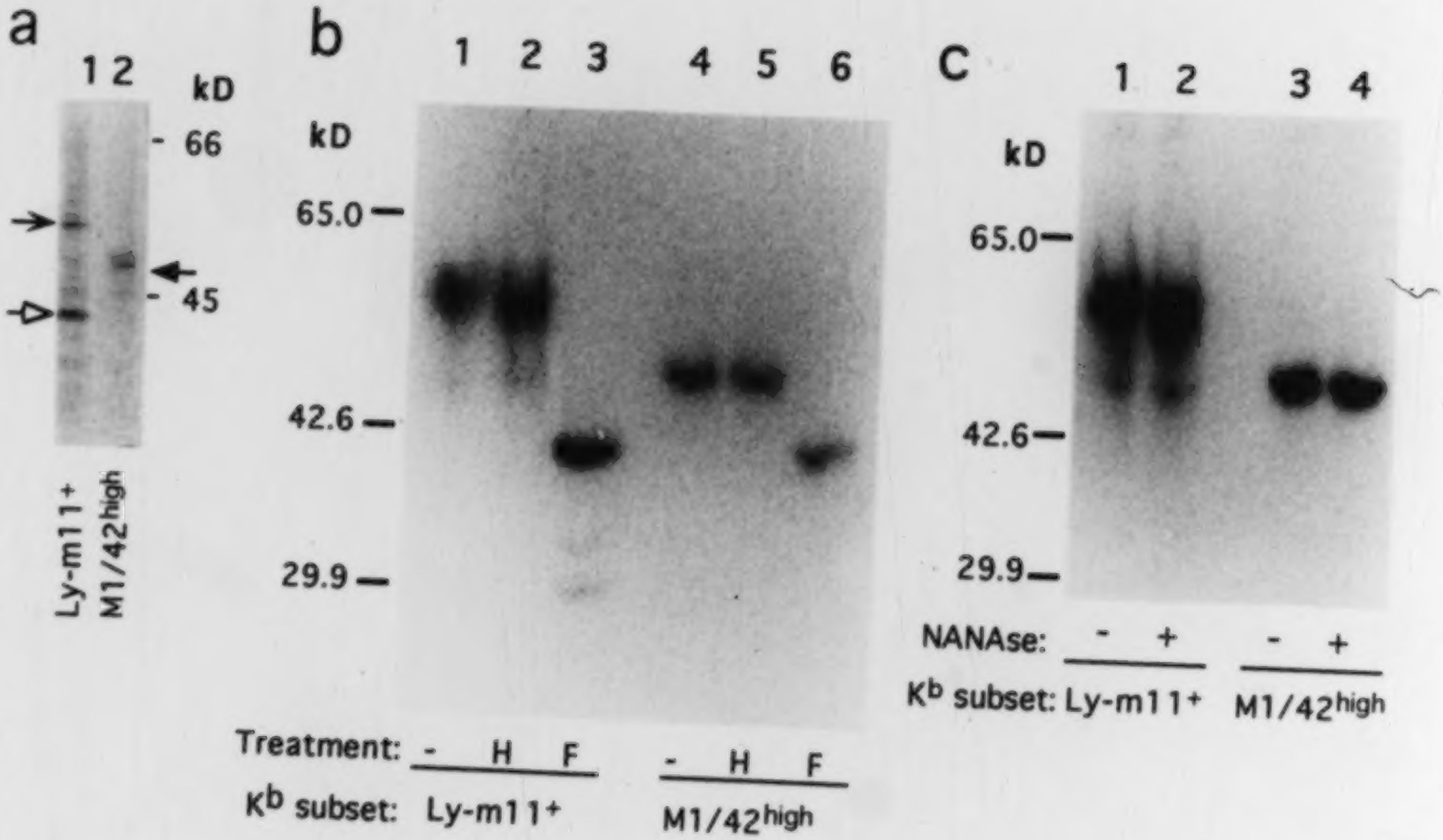


Figure 2-8. The lack of TCR recognition of Ly-m11$^+$ K^b is not due to a lack of relevant bound endogenous peptides. (A) Clone 35 killing of ^{51}Cr-labelled T2K^b cells sensitized with 10 µl of peptides isolated from both K^b subsets with equivalent Y3-reactivity. (B) Different volumes of eluted peptides isolated from Ly-m11$^+$ (□) or M1/42^{high} (◆) K^b subsets were used to sensitize T2K^b cells for killing by a K^b allo-specific CTL clone, 11. Percent specific lysis is expressed as the mean of triplicate wells.

Figure 2-9. Biochemical analysis of the Ly-m11^{+} and M1/42^{high} K^{b} subsets. (*a*) SDS-PAGE analysis of the purified K^{b} subsets. Equivalent amounts of the Y3-reactive Ly-m11^{+} (lane 1) and M1/42^{high} (lane 2) K^{b} subsets were analysed on 10% SDS-PAGE with Coomassie blue staining. Molecular weight markers are shown on the right. (*b*) Endo H and Endo F treatment of the K^{b} subset. Both K^{b} subsets were treated with 6 mU Endo H (H) or 0.8 U Endo F (F) or mock treated (-) at 37°C for 20 h, analysed on 10% SDS-PAGE and visualized by autoradiography. Molecular weight markers are as shown. (*c*) NANAse treatment of the K^{b} subsets. The K^{b} subsets were treated with 0.5 U NANAse (+) or mock treated (-) at 37°C for 4 h, analysed on 12.5% SDS-PAGE and visualized by autoradiography.


a
1 2
kD
66
45
Ly-m11+
M1/42high
b
1 2 3 4 5 6
kD
65.0
42.6
29.9
Treatment: - H F - H F
Kb subset: Ly-m11+ M1/42high
C
1 2 3 4
kD
65.0
42.6
29.9
NANAse: - + - +
Kb subset: Ly-m11+ M1/42high

CHAPTER III

AVIDITY-ENHANCED CD8 BINDING TO MHC CLASS I AND CD8 ACCESSORY FUNCTION OCCUR INDEPENDENT OF THE CD8 β CHAIN

(Most of the data presented in this chapter is submitted for publication.)

A. Introduction

Activation of $CD8^+$ CTL requires TCR interaction with MHC class I molecules bound with antigenic peptides on target cells, as well as the participation of coreceptor/accessory molecules on the surface of CTL interacting with their respective ligands on target cells (Springer, 1990). CD8 plays a critical role in CD8-dependent CTL recognition and activation. CD8 can function as a coreceptor by binding the same MHC class I molecules recognized by the TCR to aid the initiation of CTL activation (Miceli and Parnes, 1993; Julius et al., 1993). In addition, CD8 expressed on CTL can serve as an accessory molecule by binding to non-Ag class I molecules that are not interacting with the TCR, upon minimal TCR stimulation (O'Rourke et al., 1990; Kane and Mescher, 1993; O'Rourke and Mescher, 1993; Luescher et al., 1995). When the TCR is triggered, signals are generated which activate CD8 to a state of higher avidity, and consequently, the "activated", avidity-enhanced CD8 binds MHC class I molecules and facilitates CTL activation either by strengthening the interactions between CTL receptors and their ligands, or transducing additional signals for T cell activation (Anel et al., 1996).

The CD8 molecule is typically expressed as a disulfide-linked αβ heterodimer on mature murine CD8$^+$ T cells (Miceli and Parnes, 1993), however, the relative contributions of CD8 α and β chains to T cell development and activation have not been fully determined (Zamoyska, 1994). Transfection of CD8 β genes into CD8 α-expressing T cell hybridomas can further enhance IL-2 production of the T cell hybrids (Wheeler et al., 1992; Karaki et al., 1992). CD8 β gene knock-out mice display reduced numbers of mature CD8$^+$ T cells as well as impaired negative selection process (Nakayama et al., 1994; Fung-Leung et al., 1994; Crooks and Littman, 1994). Although CD8 β chain itself does not seem to be involved in signal transduction, it can assist or stabilize CD8 α chain association with p56^{lck} (Irie, et al., 1995). These observations clearly demonstrate a role of CD8 β chain in T cell development and activation.

Less clear are the relative contributions of CD8 α and β to CD8 coreceptor and accessory functions. As demonstrated by photoaffinity labeling and anti-CD8 antibody blocking, CD8 αβ heterodimers appear to be more efficient in facilitating soluble MHC class I binding to specific TCR than CD8 αα homodimers (Luescher et al., 1995; Renard et al., 1996), suggesting that the CD8 β chain can play a role in CD8 coreceptor function. However, the precise role of CD8 β chain in CD8 coreceptor function is undetermined. In addition, although CD8 αα homodimers are sufficient to mediate static-state CD8 adhesion to MHC class I (Salter et al., 1990; Garcia et al., 1996b), it is unknown whether the CD8 β chain is required for TCR-triggered, activated CD8 binding to MHC class I, since the previous experiments demonstrating activated CD8 binding were performed using CTL expressing CD8 αβ heterodimers (O'Rourke et al., 1990; Kane and Mescher, 1993; Luescher et al., 1995).

To directly determine whether CD8αα homodimers are sufficient to mediate TCR-triggered, activated CD8 binding to MHC class I molecules and enhance responses, we took advantage of a CD8αα-expressing murine T cell hybrid, B3Z, to test the ability of this T cell hybrid line, upon a suboptimal TCR stimulation, to bind immobilized MHC class I molecules and facilitate subsequent cytokine production.

B. Materials and Methods

Cell line and antibodies. The B3Z cell line (kind gift of Dr. N. Shastri, University of California, Berkeley, CA) is a fusion hybrid between murine CTL B3 (OVA/K^b specific) and Z.8 (BW5147 transfected with NFAT-*lacZ*) in which CD8 expression was restored by transfection with a CD8α cDNA (Karttunen et al., 1992). This cell line was maintained in RPMI supplemented with 10% FCS, 20 mM Hepes, 20 μM 2-ME, 1 mM L-glutamine, sodium puruvate, nonessential amino acids, and 100 μg/ml penicillin-streptomycin. Typically, B3Z was grown in selection medium containing 400 μg/ml hygromycin B (Boehringer Mannheim GmbH, Mannheim, Germany) and 1.0 mg/ml geneticin (G418; GibcoBRL, Grand Island, NY). However, B3Z hybrids grown in non-selection medium could stably maintain CD8α expression and LacZ inducibility for at least three months, and were sometimes used in experiments.

The following mAbs: anti-TCRα/β, H57.597 (Kubo et al., 1989); anti-CD3ε, 145-2C11 (Leo et al., 1987); anti-CD8α, 53.6.72 (Ledbetter and Herzenberg, 1979) and anti-CD8β, 53.5.8 (Ledbetter and Herzenberg, 1979) were purchased from PharMingen (San Diego, CA). YTS169.4, an anti-CD8 α mAb (Cobbald et al., 1984) was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). The rat anti-mouse CD8α-producing hybridoma, 2.43 (Sarmiento et al., 1980) was obtained from ATCC (Rockville, MD).

Purification of murine MHC class I molecules. The immunoaffinity purification of murine MHC class I molecules used in this study, K^k (isolated from RDM-4 cells) and D^b (isolated from EL4 cells), was as described before (Kane et al., 1989) and in Chapter II.

FACS analysis. 1 X 10^5 B3Z cells were incubated with various primary mAbs on ice for 30 min. Cells were extensively washed and stained with 1:100 dilution of FITC-conjugated goat anti-rat IgG or goat anti-hamster IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The FITC-conjugated, isotype-matched secondary mAbs alone were used as negative controls. Fluorescence intensity was measured by a FACScan instrument (Becton-Dickinson, Mountain View, CA) and analyzed with Lysis II software.

Cell binding assay. Cell binding to immobilized proteins in 96-well plates has been described in detail (Shen et al., 1996). 1 X 10^5 ^{51}Cr-labeled B3Z cells were treated with 1.0 μg/ml soluble anti-TCR mAb, H57.597, prior to loading into wells bearing purified K^k or D^b molecules at the indicated densities. After incubation at 37°C for 2 h, unbound cells were removed. Cell binding was calculated as: percent cell bound = 100 x [(cpm bound)/(total cpm-spontaneous cpm)]. In some experiments, B3Z cells were incubated with anti-CD8 mAbs (YTS.169.4 at 10 μg/ml and 2.43 used as 1:2 culture supernatant) for 30 min at RT before addition of anti-TCR mAb. All experiments were performed in triplicate. Variation between wells was less than 10%.

IL-2 assay. Purified K^k was immobilized at 0.1 μg/well, whereas anti-CD3 mAb, 2C11, was coated at 1.0 μg/well. 1 x 10^5 B3Z cells were incubated with or without soluble anti-TCR/CD3 stimulation at 1.0 μg/ml in wells coated with various proteins at 37°C for 16 h. At the end of incubation, supernatants were harvested and IL-2 concentrations were measured by sandwich ELISA following manufacturer's instructions (PharMingen). The capture and detection anti-IL-2 mAbs are JES6-1A12 (Rat IgG_{2a}) and biotinylated JES6-5H4 (Rat IgG_{2b}), respectively. The IL-2 concentrations were calculated using a standard curve obtained with recombinant mouse IL-2. All determinations were carried out in triplicate wells. Results are expressed as mean IL-2 concentration (ng/ml) ± SD.

LacZ assay. B3Z LacZ activities at various culture conditions were measured simultaneously with the IL-2 assay described above. At the end of incubation, supernatants were carefully harvested for IL-2 determination, while the remaining cells were used for bulk LacZ assays by chromogenic LacZ substrate, chlorophenol red β-galactoside (CPRG; Calbiochem, San Diego, CA) as described (Sanderson and Shastri, 1994). Individual wells were gently washed with 100 µl PBS, and cells were lysed by addition of 100 µl Z buffer (100 mM 2-ME, 9 mM $MgCl_2$, 0.125% NP-40 in PBS) containing 0.15 mM CPRG. After incubation at 37°C for 4 h, 50 µl of stop buffer (300 mM glycine and 15 mM EDTA in H_2O) was added to each well and OD_{570nm} was determined using a 96-well plate reader. All determinations were carried out in triplicate wells, and results are expressed as mean OD_{570nm} ± SD.

C. Results

FACS analysis of the T cell hybrid B3Z. The B3Z cell line is a T cell hybrid between CTL B3 (OVA/K^b specific) and Z.8 (BW5147 transfected with NFAT-*lacZ*) in which CD8 expression was restored by transfection with a CD8α cDNA (Karttunen et al., 1992). This hybrid can be activated by specific Ag to express *LacZ* and produce IL-2 in a CD8 dependent manner (Karttunen et al., 1992). FACS analysis showed that the B3Z we examined here expresses CD8 α, but no β chains (Figure 3-1, upper panel). As reported, the B3Z expresses TCR/CD3 complexes (Figure 3-1, lower panel). This has allowed us to use this T cell hybrid to directly test whether CD8 αα expression alone, is sufficient to mediate avidity-enhanced CD8 binding to MHC class I molecules, resulting from a TCR stimulus.

Soluble anti-TCR mAb triggers B3Z (CD8αα⁺) hybrid cells to bind purified, immobilized MHC class I molecules and the binding is inhibited by anti-CD8 mAbs. By using CD8αβ-expressing CTL clones and a solid phase system, we and others have demonstrated that soluble anti-TCR/CD3 mAb (a suboptimal stimulus) is not sufficient to fully induce CTL responses, but can trigger CD8αβ⁺ CTL to bind immobilized MHC class I molecules, and consequently enhance a CTL degranulation response (O'Rourke et al., 1990; Kane and Mescher, 1993; Shen et al., 1996). Here, we tested whether soluble anti-TCR mAb can trigger CD8αα-expressing B3Z T cell hybrids to bind immobilized, purified MHC class I molecules. As shown in Figure 3-2 A, soluble anti-TCR mAb, H57.597, triggered B3Z to bind immobilized murine class I molecules, K^k and D^b. The levels of binding were comparable or slightly lower than that of CD8αβ-expressing CTL (Shen et al., 1996 and Chapter II). The TCR-triggered binding is dependent on the density of class I molecule input (Figure 3-2 B). Importantly, the B3Z TCR-triggered binding to immobilized class I could be significantly inhibited by anti-CD8α mAbs (Figure 3-2 B), indicating that CD8α is mediating this avidity-enhanced CD8 adhesion.

B3Z (CD8αα⁺) IL-2 production and LacZ expression in response to soluble anti-TCR/CD3 mAbs is observed in the presence of immobilized class I. We also tested whether, under suboptimal TCR/CD3 stimulation conditions, the activated CD8αα binding to class I can enhance B3Z T hybrid cell responses as observed for activated CD8αβ of CTL (O'Rourke et al., 1990; Kane and Mescher, 1993; Shen et al., 1996). The B3Z cell line carries a construct containing transcription factor, NFAT, binding sites of the IL-2 promoter region linked to a bacterial LacZ reporter gene (Karttunen and Shastri, 1991). Stimulation of B3Z cells with anti-CD3 mAb or specific antigen results in the production of IL-2 and the induction of LacZ activity (Karttunen et al., 1992). It has been shown that B3Z cell IL2 production and LacZ activation are dependent on CD8 participation (Karttunen et al., 1992). We measured IL-2 secretion and LacZ activity of B3Z cells under various culture conditions. As shown in Figure 3-3, when B3Z cells were cultured with immobilized BSA, non-Ag K^k class I molecules, or with soluble anti-TCR mAb alone, no IL-2 secretion was observed. In contrast, when B3Z cells were treated with soluble anti-TCR (H57.597) or anti-CD3 (2C11) and incubated on immobilized K^k molecules, significant IL-2 production was detected, at levels above 50% of that observed with immobilized anti-CD3 mAb (Figure 3-3 A). Since the B3Z cell line contains the inducible NFAT-*LacZ* construct, B3Z β-galactosidase activity was also induced in a similar pattern to IL-2 secretion (Figure 3-3 B) (Karttunen et al., 1992; Sanderson and Shastri, 1994). These results indicated that CD8αα homodimers are functionally competent to mediate TCR-triggered binding to class I and resulting costimulation.

D. Concluding Remarks

Using a T cell hybrid, B3Z, which expresses only CD8 αα homodimers, I have directly demonstrated that TCR-triggered, activated CD8 binding to MHC class I can occur efficiently independent of the CD8 β chain. The activated CD8αα binding to class I also enhances the T cell response to a suboptimal TCR stimulus. The potential role(s) of CD8 β chains in this type of accessory CD8-MHC class I interaction remains to be elucidated, however, since CD8 αα homodimers are sufficient to mediate avidity-enhanced binding to MHC class I, any role(s) of CD8 β chain in modulating this type of CD8-class I interaction is likely to be subtle. Alternatively, the primary function of the CD8 β chain may be to enhance CD8 low-affinity coreceptor function(s) (Zamoyska, 1994; Garcia et al., 1996b; Shen et al., 1996; Renard et al., 1996 a & b).

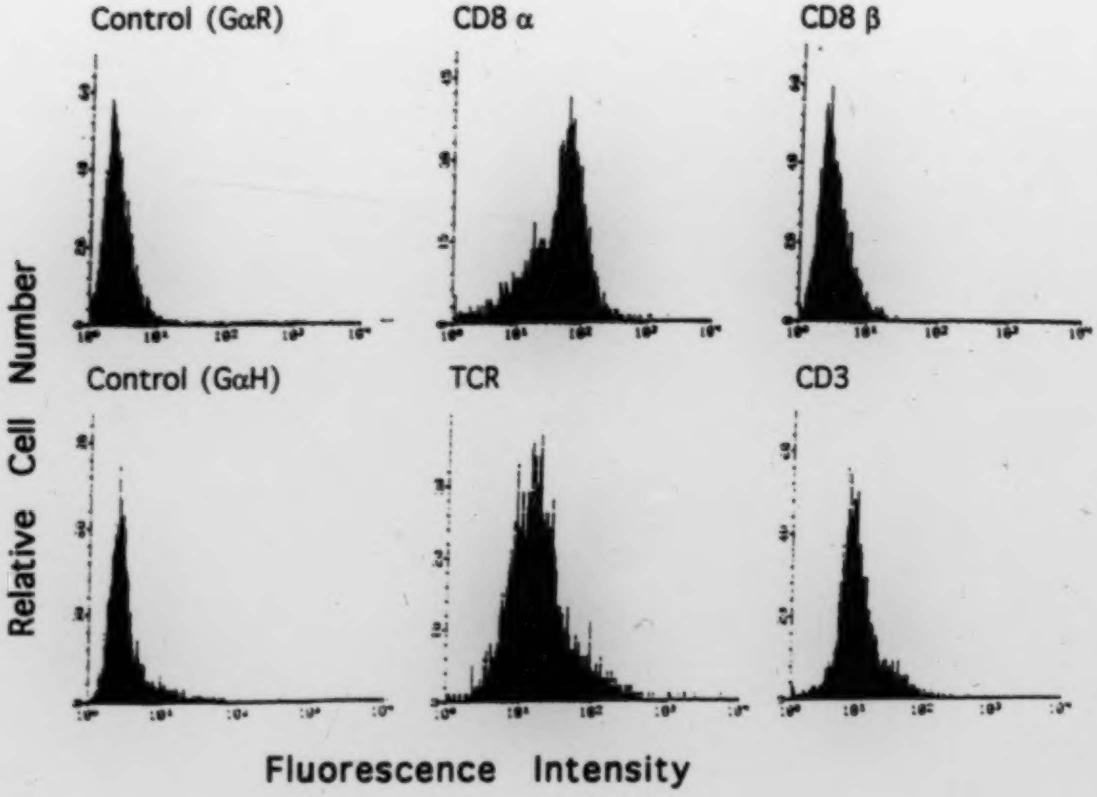


Figure 3-1. FACS analysis of the T cell hybrid B3Z. Upper panel: B3Z expresses CD8 α chain, but not CD8 β chain. Lower panel: B3Z expresses TCR/CD3 complexes. 1 X 10^5 cells were stained with indicated 1st-Ab, 53-6.7 for CD8α, 53-5.8 for CD8β, H57 for TCR and 2C11 for CD3ε. Cells were then stained with FITC-conjugated goat anti-rat IgG (GαR) or goat anti-hamster IgG (GαH). Fluorescence intensity was measured by FACScan.

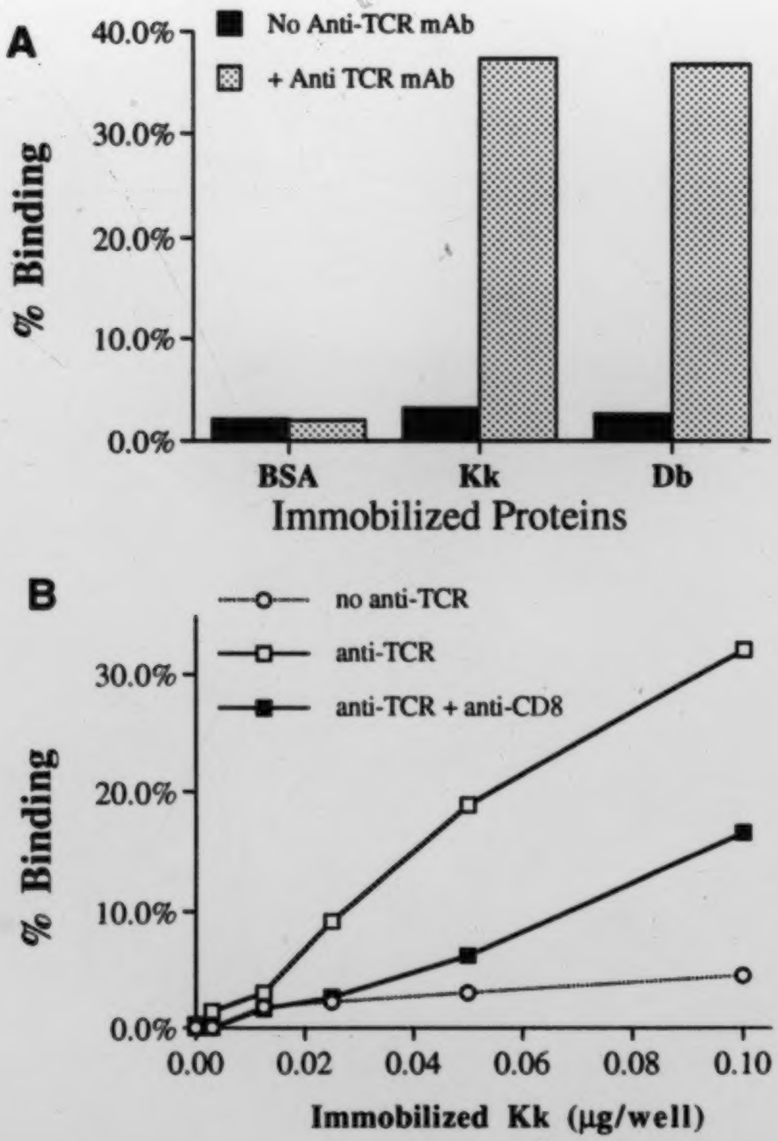


Figure 3-2. Soluble anti-TCR mAb triggers B3Z ($CD8\alpha\alpha^+$) hybrid cells to bind purified, immobilized MHC class I molecules and the binding is inhibited by anti-CD8 mAbs. **A.** Purified K^k or D^b were immobilized on the plastic wells at a density of 0.1 μg/well. Control wells were bound with BSA. 1 X 10^5 ^{51}Cr-labeled B3Z cells were treated with 1.0 μg/ml soluble anti-TCR mAb, H57.597, immediately before loading into the wells. **B.** Purified K^k were immobilized on the plastic wells at the indicated densities. B3Z cells were incubated with anti-CD8 mAbs (YTS.169 at 10 μg/ml and 2.43 used as 1:2 culture supernatant) before addition of anti-TCR mAb, H57.597. In both A and B, after incubation at 37°C for 2 h, unbound cells were removed and the percentage of cell binding was calculated.

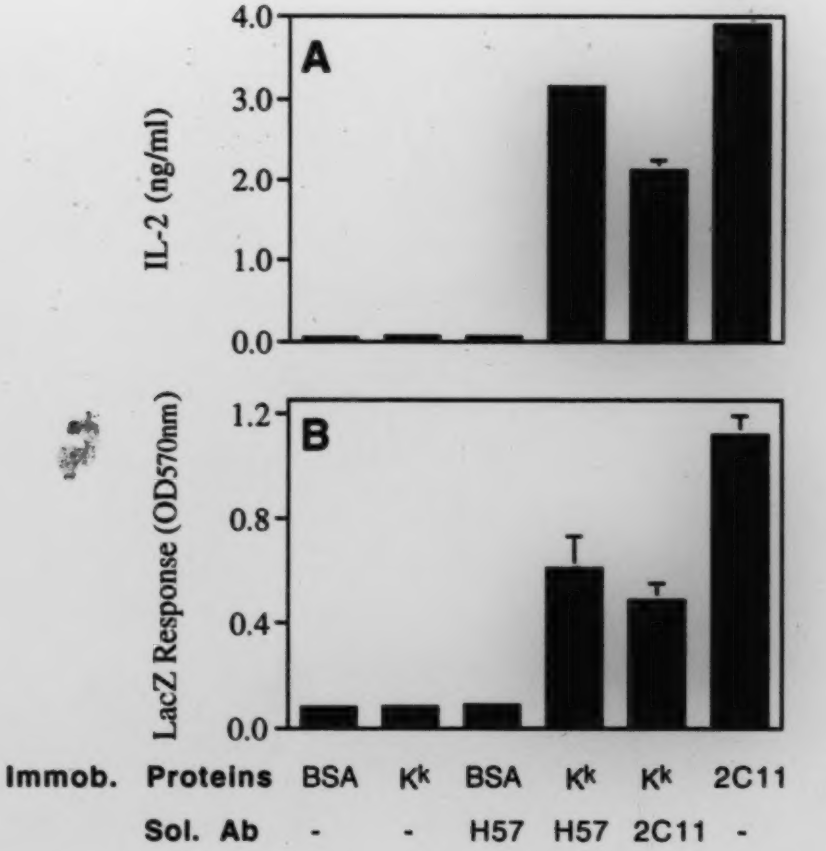


Figure 3-3. B3Z ($CD8\alpha\alpha^+$) IL-2 production (A) and LacZ activity (B) in response to soluble anti-TCR/CD3 mAbs is observed in the presence of immobilized class I. Purified K^k was immobilized at 0.1 μg/well, whereas anti-CD3 mAb (2C11) was coated at 1.0 μg/well. 1 x 10^5 B3Z cells were incubated with or without soluble anti-TCR/CD3 stimulation in wells coated with the indicated proteins at 37°C for 16 h. Both soluble H57.597 (anti-TCR mAb) and 2C11 (anti-CD3 mAb) were used at 1.0 μg/ml. Supernatants were harvested and IL-2 concentrations were measured by sandwich ELISA; Bulk LacZ activities of the remaining B3Z cells were measured by CPRG colorimetric assay. Results represent the mean ± SD.

CHAPTER IV

GLU227->LYS SUBSTITUTION IN THE ACIDIC LOOP OF MHC CLASS I α3 DOMAIN DISTINGUISHES LOW AVIDITY CD8 CORECEPTOR AND AVIDITY-ENHANCED CD8 ACCESSORY FUNCTIONS

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A. Introduction

Cytotoxic T lymphocyte activation is a process that involves multiple cell surface protein interactions with antigen bearing target cells and a cascade of signal transduction events which lead to proliferation and effector functions (Springer, 1990; Collins et al., 1994). T cell receptor interaction with MHC class I molecules bound with antigenic peptides on target cells is responsible for the specificity of $CD8^+$ CTL recognition. So-called coreceptors and accessory molecules on the surface of CTL interact with their respective ligands on target cells and also contribute to T cell activation, particularly as in most circumstances where antigen density is limiting on the targets (Springer, 1990; Collins et al., 1994). Coreceptor and accessory molecules contribute to T cell activation by increasing the avidity between CTL and targets, amplifying TCR-initiated signals, transducing distinct signals, or a combination of these mechanisms (Springer, 1990; Collins et al., 1994).

The CD8 molecules plays a critical role in CD8-dependent CTL recognition and activation. The CD8 molecule can serve as a coreceptor in that it binds to the same MHC class I molecules as the TCR to facilitate efficient TCR signaling (Potter et al., 1987; Julius et al., 1993; Miceli and Parnes, 1993). Based on anti-CD8 blocking experiments, Swain (1983) proposed that CD8 plays an important role (albeit not always obligatory) in T cell responses to MHC class I antigen, and serves as an "facilitator" by interacting with the non-polymorphic region on MHC class I. More direct evidence came from CD8 gene transfer experiments. Introduction of CD8 genes into T cell hybridomas enhances T cell cytolytic activity (Dembic et al., 1987) as well as IL-2 production (Gabert et al., 1987; Wheeler et al., 1992).

Potter et al. (1983) isolated, by immunoselection, H-2^d somatic mutant cells that lost the 34-2-12 epitope expression in the $\alpha3$ domain of D^d, while retaining all the serologically defined $\alpha1$ and $\alpha2$ D^d determinants. These mutant cells did not serve as targets for D^d allogeneic CTL as their parental cells, Later, it was shown (Potter et al., 1987) the D^d expressed on the mutant cells had an acid to base single amino acid substitution in the $\alpha3$ domain, i.e. the glutamic acid at residue 227 substituted by a lysine; and this substitution is responsible for the abrogation of 34-2-12 mAb reactivity. It was further demonstrated that cells transfected with the mutant D^d did not serve as allo-CTL targets and failed to elicit a primary *in vitro* CTL response (Potter et al., 1987; Connolly et al., 1988). It was observed that only the CD8-dependent, but not the CD8-independent allo-CTL failed to lyse cells expressing the Glu227->Lys D^d (Connolly et al., 1988; Potter et al., 1989). Transgenic mice expressing the $\alpha3$ domain mutant class I failed to eliminate self-reactive T cells, suggesting a role of class I $\alpha3$ domain interaction with CD8 in thymocyte negative selection process (Ingold et al., 1991). These functional data demonstrate a crucial role of CD8 interaction with class I $\alpha3$ domain in which Glu227 is critically involved.

The above structure-function analysis suggested a role for the class I α3 domain in recognizing CD8, however, it did not actually show that CD8 binds MHC class I and that the α3 domain mutation in class I affects CD8 binding. It has been directly demonstrated that CD8 transfectants (Norment et al., 1988) and CD8-incorporated liposomes (Rosenstein et al., 1989) bind to class I expressing cells or vesicles. Using a similar cell-cell binding assay combined with site-directed mutagenesis, it has been further shown that the negatively charged loop (residue 222 - 229) in the highly conserved α3 domain of MHC class I molecules is a binding site for CD8 (Salter et al., 1989 & 1990). The substitutions in the MHC class I α3 domain abrogated the CD8-dependent CTL killing of target cells expressing these mutant molecules (Salter et al., 1989 & 1990; Connolly et al., 1990; Sakemata et al., 1993). The inability of CD8-dependent CTL to kill targets bearing the α3 mutated class I molecules was attributed to the disruption of CD8 interaction with class I (Potter et al., 1987; Connolly et al., 1990; Salter et al., 1990). In summary, these data strongly indicate that the acidic loop in the MHC class I α3 domain is a critical site for CD8 interactions, in which CD8 is most likely functioning as a coreceptor.

In addition to serve a coreceptor, CD8 molecules can be "activated" or "triggered" by TCR stimulation to bind to class I molecules with high avidity, including class I not recognized by the TCR as antigenic complexes (i.e. non-Ag class I), to augment CTL responses and thus serve an accessory molecule function (O'Rourke et al., 1990; O'Rourke and Mescher, 1993; Kane and Mescher, 1993; Takeshita et al., 1993; Luescher et al., 1995). The site(s) on the MHC class I molecules that participate in this high avidity TCR-activated CD8 binding has not been identified. Although the acidic loop in class I α3 domain is critical for non-activated CD8αα binding as shown by static-state binding assays (Salter et al., 1990), whether this acidic loop is also essential for "TCR-activated" CD8αβ heterodimer binding to class I is unknown. Recent mutagenesis data showing that mutations in the $\alpha2$ domain of HLA class I molecules interfere with CD8$\alpha\alpha$ binding suggest the possibility that other sites in addition to the $\alpha3$ domain on class I may be involved in CD8 interaction(s) (Giblin et al., 1994; Sun et al., 1995), perhaps including activated CD8 binding. The goals of this study are to directly determine 1) whether the Glu227 in class I $\alpha3$ domain is necessary for activated CD8 binding; and 2) how class I Glu227->Lys substitution affects CD8 serving coreceptor versus accessory functions.

It has been well established that purified MHC class I molecules can efficiently form stable complexes with exogenous antigenic peptides on plastic or Latex beads, to stimulate responses of Ag-specific CTL (Kane et al., 1989c; Kane and Mescher, 1993) and T cell hybrids (von Bonin et al., 1993; Stryhn et al., 1994). To employ this solid phase system, we first purified chimeric class I molecules consisting of $D^b\alpha1\alpha2$ or $K^b\alpha1\alpha2$ and $D^d\alpha3$ domains, in which the $D^d\alpha3$ domain is either the wild type or the mutant with a Glu227 ->Lys substitution. To directly test the ability of the $\alpha3$ domain wild type or mutant class I to serve as ligands for activated CD8, the purified wild type or the mutant class I molecules are immobilized on the plates, and tested for their ability to mediate TCR-activated CD8-dependent CTL binding and responses. In a complementary manner, to test the ability of the wild type and mutant class I to serve a CD8 coreceptor ligand, the purified wild type and mutant molecules are complexed with antigenic peptide, and compared for their abilities to trigger CTL binding and responses to defined, coimmobilized non-Ag class I molecules.

B. Materials and Methods

mAbs. A murine hybridoma which secrets an IgG2_a mAb recognizing the $\alpha1$ domain of H-2 D^b molecules, B22.249 (Lemke et al., 1979), was a gift from Dr. U. Hammerling (Memorial Sloan-Kettering Cancer Center, New York). The K^b-specific mAb, Y3 (IgG2_b) (Hammerling et al., 1982), and D^d-specific mAbs, 34-5-8s (IgG2_a) (Ozato et al., 1982) and 34-2-12s (IgG2_a) (Ozato et al., 1982) were produced from their respective hybridomas obtained from American Type Culture Collection (ATCC; Rockville, MD). Rat anti-CD8 mAb, 2.43 (IgG2_b) (Sarmiento et al., 1980) was obtained from ATCC and used as culture supernatant. Other anti-CD8 mAbs, YTS169.4 (Rat IgG2_b) (Cobbald et al., 1984) were purchased from Cedarlane Laboratories (Hornby, Ontario, Canada), and 53.6.72 (Rat IgG2_a) (Ledbetter and Herzenberg, 1979), from Pharmingen (San Diego, CA). The hamster anti-mouse TCR$\alpha\beta$ mAb, H57-597 (Kubo et al., 1989), was also purchased from Pharmingen.

Transfectant cell lines expressing chimeric H-2 class I with wild type or mutant $\alpha3$ domains. M12 (H-2^d), a B lymphoma cell line, was transfected with chimeric class I genes (Potter et al., 1989; Knall et al., 1994 & 1995). Exon shuffled genes consisting of the $\alpha1$ and $\alpha2$ domain of the H-2 D^b or K^b genes and either the wild type or Glu227->Lys substituted $\alpha3$ domain of the H-2 D^d (Potter et al., 1989) were transfected into the M12 cell line by electroporation, and are referred to herein as M12.D^b/D^dwt, M12.D^b/D^dLys, M12.K^b/D^dwt and M12.K^b/D^dLys, respectively. These stable tranfectants were kindly supplied by Dr. T. Potter, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO, and were maintained in 5% FCS-DMEM containing 10 mM Hepes and 100 µg/ml penicillin-streptomycin.

Cloned CTLs. Clone 3/4 is specific for H-2 D^b and the influenza nucleoprotein (NP) (365-380) of A/PR/8/34 influenza virus (Kane and Mescher, 1993). It was derived from 6- to 8-week old C57BL/6 mice by i.p. immunization with 300 hemagglutination U of the influenza viruses as infectious allantoic fluid. Six weeks after immunization, spleen cells from primed mice were restimulated *in vitro* with irradiated A/PR/8/34-infected C57BL/6 spleen cells and subsequently cloned by limiting dilution. It was maintained by weekly stimulation with NP peptide-pulsed C57BL/6 spleen cells. The NP peptide used in this study is a 10-mer (NP366-374) with a tyrosine at the N terminus (Y-ASNENMETM) which was synthesized and purified at Multiple Peptide Systems (San Diego, CA). The clone was cultured in 10% FCS-RPMI 1640 supplemented with 20 mM Hepes, 2 mM L-glutamine, 5×10^{-5} M 2-ME, 100 µg/ml penicillin-streptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 15 U/ml recombinant human IL-2.

Purification of chimeric murine MHC class I molecules bearing wild type or mutant α3 domains. The chimeric H-2 class I molecules, D^b/D^dwt, D^b/D^dLys, K^b/D^dwt and K^b/D^dLys were purified from their respective transfectant cells by immunoaffinity chromatography as described (Kane et al., 1989a; Shen and Kane, 1995) with modifications. The transfectant cells expressing wild type or mutant α3 domain chimeric molecules were grown in 5% FCS-DMEM and, typically, 0.5 - 1.0 x 10^{10} cells were used for each purification. Since B22.249 mAb recognizes the α1 domain of D^b (Allen et al., 1984), and Y3 mAb recognizes the α2 domain of K^b (Ajitkumar et al., 1988), these mAbs were coupled to Sepharose-4B beads and used for immunoaffinity purifications of D^b/D^d and K^b/D^d chimeric molecules, respectively. To avoid cross contamination, separate columns were employed for the wild type and the mutant class I molecules. Detergent lysates of the transfectant cells were passed over the B22.249 or Y3 columns. Columns were washed with 0.1% DOC, 10 mM NaCl, 10 mM phosphates, pH 8.2, and 0.5% DOC, 0.65 M NaCl, 10 mM Tris, pH 8.5. The chimeric molecules were then eluted from the columns by using 0.5% DOC, 0.15 M NaCl, 15 mM Na_2CO_3, pH 10.5 (Shen and Kane, 1995). Solid-phase ELISA of affinity-purified H-2 class I chimeric molecules was performed with various mAbs as described in Chapter II. Peak fractions showing strong ELISA reactivities were pooled and used. The protein quantitation of purified class I molecules was determined by Micro-BCA Assay (Pierce, Rockford, IL). Other immunoaffinity purified H-2 class I used in this study, including K^b and D^b isolated from EL4 cells and K^k isolated from RDM-4, as well as $I\text{-}E^d$ class II molecules isolated from A20.Cy, were purified as described previously (Kane et al., 1989a; Kane, 1994).

Peptide pulsing of H-2 class I-bearing cells and purified class I molecules. For experiments involving peptide-pulsed cells, the cells were resuspended in 2% FCS-RPMI and incubated with NP peptide at the desired concentrations at RT for 30 min. After pulsing, the cells were washed and resuspended at the desired density for various experiments.

Purified class I were diluted into D-PBS to the desired density and 100 µl was loaded into wells of the microtiter plates. After incubation at RT for 1.5 h, the wells were washed 3 times with 1 mg/ml BSA in D-PBS and then blocked with the same solution for 30 min. Then the class I-bearing wells were incubated with NP peptide resuspended in 2% FCS-PBS at 37°C for 16 h to form class I/peptide antigen complexes.

Assays for CTL binding and degranulation. Degranulation by the CTL was assessed by measuring the serine esterase (SE) activity released into the medium by BLT assay as described in Chapter II. Stimulator cells were pulsed with the NP peptide for 30 min at RT before the assay. The stimulators were then washed 3 times with 2% FCS-RPMI, and 3 x 10^5 of the stimulator cells were incubated together with 1 x 10^5 cloned CTL for 4 h. 20 µl of supernatant was recovered and the BLT reaction was allowed to proceed for 20 min whereupon the OD_{405nm} was determined. SE release is calculated as ΔOD_{405nm} = OD_{405nm}(CTL + stimulators) - OD_{405nm}(CTL alone).

CTL adhesion and degranulation responses to purified class I and coimmobilized proteins were essentially identical as detailed in Chapter II. Either 1 or 2 x 10^5 ^{51}Cr-labelled CTL were incubated for 4 h at 37°C on protein bearing plate wells and unbound cells were then removed. In experiments employing fluid phase anti-TCR mAb, the antibody was added to CTL in suspension and the cells immediately placed into the class I-bearing wells. Cell binding was calculated as: percent specific cell bound=100 x [(cpm bound)/(total cpm-spontaneous cpm)]. Degranulation by the CTL was determined simultaneously with the CTL binding from the same wells. SE release is expressed as ΔOD_{405nm} = OD_{405nm}(CTL + immobilized class I) - OD_{405nm}(CTL + wells blocked with 2% FCS in PBS). The anti-CD8 mAb blocking experiments were performed by incubating CTL with CD8-specific antibodies at RT for 30 min before carrying out the experiments. Anti-CD8 mAb, 2.43 was used at dilution 1:4 of the culture supernatant and purified YTS169.4 was used at 5 µg/ml. All the determinations were done in triplicate for each condition unless specified.

Cytotoxicity assay. Target cells were first labeled with ^{51}Cr and in some cases pulsed with NP peptide at the concentrations indicated. After washing, the target cells were diluted to 1 x 10^5/ml with assay medium and 100 μl of target cell suspensions were incubated with CD8-dependent allo-reactive CTL clones or the NP-specific CTL clone 3/4 at various E:T ratio for 5 h in V-bottom microtiter plates in triplicate. Percent specific ^{51}Cr release was calculated as: [(experimental release - spontaneous release)/(maximum release - spontaneous release)] X 100. Results are expressed as mean % specific ^{51}Cr release ± SD. In all experiments, the spontaneous ^{51}Cr release was less than 7.5% of the total unless specified. For anti-CD8 mAb blocking experiments, the cloned CTL were incubated with the indicated mAb at RT for 30 min before use in the assays. Anti-CD8 mAb, 2.43 was used at dilution 1:4 of the culture supernatant while the purified YTS169.4 and 53.6.72 were used at 5 μg/ml.

C. Results

Antigen-specific, CD8-dependent CTL recognition and lysis of target cells bearing MHC class I with wild type or mutant α3 domains. It has been shown that CD8-dependent CTL failed to kill target cells with substitutions of residues in the region of 222-229 of the α3 domain of MHC class I molecules (Potter et al., 1987 & 1989; Connolly et al., 1990; Salter et al., 1990; Sakimata et al., 1993). The inability of CTL to lyse these targets was attributed to the failure of CD8 to bind the mutated MHC class I molecules on the target cells. It was found that about 100-fold more peptide Ag was needed to sensitize target cells expressing MHC class I with a mutant α3 domain for killing by peptide-specific CTL (Knall et al., 1994). In the present study, the ability of an H-2 D^b-restricted, NP specific CTL, clone 3/4, to kill target cells transfected with chimeric cDNA consisting of D^b $\alpha1\alpha2$ domain and D^d $\alpha3$ domain was examined. The D^d $\alpha3$ domain is either the wild type or mutant with a Glu227->Lys substitution (Potter et al., 1989; Knall et al., 1994 & 1995). Since the parental cell line is M12, the transfectant cells expressing the wild type or $\alpha3$ mutated chimeric class I molecules are herein called M12.D^b/D^dwt or M12.D^b/D^dLys, respectively. Both the transfectants express comparable levels of the hybrid D^b molecules as detected by FACS analysis using a D^b-specific mAb, B22.249 (Table 4-1). The target cells were pulsed with NP peptide at different concentrations, and cytolysis was measured by ^{51}Cr release. As expected, M12.D^b/D^dwt target cells were lysed by clone 3/4 to a level comparable to EL4 (H-2^b), which expresses natural D^b molecules (Fig. 4-1 A). This indicated that the chimeric molecules of $D^b\alpha1\alpha2$ and $D^d\alpha3$ triggered lysis comparable to that of natural D^b molecules, similar to that previously reported (Knall et al., 1994). Clone 3/4 also lysed M12.D^b/D^dLys, with two significant differences compared to M12.D^b/D^dwt lysis (Fig. 4-1 A). First, the maximal levels of lysis of M12.D^b/D^dLys (20%) were only one third to one half of that of M12.D^b/D^dwt (40 - 50%). Second, the concentration of peptide required to obtain significant lysis was 10 to 100 fold higher in M12.D^b/D^dLys than in M12.D^b/D^dwt. These results are consistent with a previous report in which lysis by a K^b-restricted, OVA-specific CTL clone of M12 cells transfected with K^b/D^dLys gene was substantially lower and required higher concentrations of OVA peptide than target cells transfected with K^b/D^dwt gene (Knall et al., 1994).

The ability of anti-CD8 Ab blocking of clone 3/4 CTL killing of both targets was examined (Fig. 4-1 B and C). M12.D^b/D^dwt cell lysis by clone 3/4 CTL was almost completely blocked by CD8α-specific mAbs 2.43 and YTS169.4 at lower peptide concentrations, while partially inhibited when peptide concentrations were high (Fig. 4-1 B). This confirmed that the clone 3/4 killing is

CD8-dependent, and also showed that CD8 dependency is related to the numbers of MHC class I-peptide complexes involved in the CTL-target interactions (Alexander et al., 1991; Al-Ramadi et al., 1995). Interestingly, even the cytolysis of M12.D^b/D^dLys was partially blockable by anti-CD8 mAbs (Fig. 4-1 C) suggesting that CD8 may still participate to some extent in CTL recognition of mutant class I-peptide complexes. The residual CTL activity may be the result of high TCR occupancy resulting from interaction of MHC-peptide complexes and/or the participation of non-class I accessory molecule interactions.

It is not known to what extent the Glu227->Lys substitution in the $\alpha3$ domain of MHC class I molecules on stimulator cells affects Ag-specific CTL degranulation responses. Both M12.D^b/D^dwt and M12.D^b/D^dLys transfectants were pulsed with NP peptide, and their abilities to trigger NP-specific clone 3/4 degranulation responses were examined by the measurement of SE release (Fig. 4-2). Similar to the cytolysis results, M12.D^b/D^dwt cells triggered a substantially stronger SE release than M12.D^b/D^dLys cells, and these responses were peptide dose-dependent (Fig. 4-2 A). Clone 3/4 degranulation responses triggered by both M12.D^b/D^dwt and M12.D^b/D^dLys cells were CD8-dependent, as the anti-CD8 mAbs effectively inhibited the degranulation responses (Fig. 4-2 B and C). It is also found that SE release was more readily blocked by anti-CD8 mAbs than cytolysis at high peptide concentrations. This could suggest that the SE release is somewhat more CD8-dependent than the cytolytic response. Alternatively, it could be a reflection of the somewhat higher concentration of peptide required to detect SE release responses.

CD8-dependent allo-reactive CTL recognition and lysis of target cells bearing MHC class I with wild type or mutant $\alpha3$ domains. Lysis of M12 cells expressing chimeric K^b/D^d molecules with either the wild type (M12.K^b/D^dwt) or mutated (M12.K^b/D^dLys) $\alpha 3$ domains by several K^b allo-specific CTL clones were also determined. Both the M12.K^b/D^dwt and M12.K^b/D^dLys cells expressed comparable levels of the chimeric K^b molecules. Lysis of the M12.K^b/D^dwt target cells was between 20 to 40% by K^b allo-specific CTL clone AB.1 (Fig. 4-3 A), and clone 11 (Fig. 4-3 B). However, no killing of M12.K^b/D^dLys targets by clone 11 (Fig. 4-3 B), and only low level of lysis (1/6 to 1/3 of M12.K^b/D^dwt cells) by clone AB.1 was observed (Fig. 4-3 B). These results are consistent with the previous reports (Potter et al., 1987 & 1989; Connolly et al., 1990; Salter et al., 1990), that the class I $\alpha 3$ domain is critical for CD8-dependent allo-CTL recognition. Since the antigenic peptides involved in these allo-recognitions have not been identified, in the following studies, we focus on the antigen-specific CTL recognition in which the class I/peptide complexes can be formed in a controlled manner.

Immunoaffinity purification of chimeric class I D^b and K^b molecules containing the wild type or mutant $\alpha 3$ domains. FACS analysis showed that both M12.D^b/D^dwt and M12.D^b/D^dLys transfectant cells expressed 34-5-8 epitopes (D^d $\alpha 1 \alpha 2$) (Table 4-1), suggesting that they both expressed parental D^d class I molecules. In addition, they may express other accessory molecule ligands on their cell surfaces. In order to accurately assess and directly compare the role(s) of D^b/D^dwt- and D^b/D^dLys-peptide complexes in CTL recognition and activation, we purified D^b/D^dwt and D^b/D^dLys molecules for use in solid-phase CTL recognition systems (Kane et al., 1989c; Kane et al., 1993; Shen and Kane, 1995). Since B22.249 mAb recognizes an $\alpha 1$ domain epitope of D^b (independent of $\alpha 3$ domain) (Allen et al., 1984), it was used for immunoaffinity purification of D^b/D^dwt and D^b/D^dLys chimeric molecules. The D^b/D^dwt (Fig. 4-4 A) and D^b/D^dLys (Fig. 4-4 B) chimeric molecules can be efficiently eluted from B22 affinity columns. The K^b/D^dwt and K^b/D^dLys class I molecules were also purified from M12.K^b/D^dwt and M12.K^b/D^dLys cell detergent lysates, respectively, using separate Y3 affinity columns (Fig. 4-4 C and D). The purified D^b/D^dwt (pD^b/D^dwt) and pD^b/D^dLys (pD^b/D^dLys) were analysed using solid-phase ELISA (Kane et al., 1989a) (Table 4-2). Both pD^b/D^dwt and pD^b/D^dLys were strongly positive for the B22.249 epitope (D^b $\alpha 1$); while negative for the 34-5-8s epitope (D^d $\alpha 1 \alpha 2$) indicating the purified chimeric D^b did not contain any natural D^d molecules. As expected, the pD^b/D^dwt, but not the pD^b/D^dLys, was reactive with 34-2-12s (D^d $\alpha 3$) since the D^d Glu227 -> Lys substitution abolishes recognition by the 34-2-12s mAb (Potter et al., 1989; Connolly et al., 1990). All preparations were standardized by protein and ELISA assays.

Direct comparison of CTL adhesion and response to purified D^b/D^dwt and D^b/D^dLys pulsed with NP peptide Ag. Both pD^b/D^dwt and pD^b/D^dLys were immobilized separately on microtiter plate wells, pulsed with NP peptide at the concentrations indicated and tested for their abilities to trigger H-2 D^b-restricted, NP-specific CTL (Fig. 4-5). H-2 D^b molecules (pD^b) isolated from EL4 cells pulsed with NP peptide were also included in parallel. The adhesion as well as degranulation responses of the NP-specific CTL (clone 3/4) triggered by the immobilized, class I-NP peptide complexes were examined. As shown previously (Kane et al., 1989c; Kane and Mescher, 1993), clone 3/4 bound to, and degranulated in response to, the immobilized pD^b-NP peptide complexes (Fig. 4-5). Clone 3/4 cells bound to both pD^b/D^dwt and pD^b/D^dLys, but the levels of binding and response are dramatically different (Fig. 4-5). We consistently observed that the maximal percentage of clone 3/4 CTL cells adhered to immobilized pD^b/D^dwt was more than 3-fold greater than to pD^b/D^dLys (38% compared to 10%). CTL binding to both pD^b/D^dwt and pD^b/D^dLys was peptide dose-dependent and reached plateau at 10 µM NP peptide which probably represents a peptide concentration resulting in saturation of peptide binding by the immobilized class I molecules. CTL SE release response showed very similar results (Fig. 4-5 B). The degranulation response as detected by SE release was also peptide dose-dependent and maximal degranulation was a minimum of 3 to 5 times stronger to pD^b/D^dwt than pD^b/D^dLys.

The pD^b/D^dwt and pD^b/D^dLys density dependence for CTL binding and degranulation was also investigated (Fig. 4-6). Both pD^b/D^dwt and pD^b/D^dLys were coated separately at several densities on the plastic and pulsed with a saturating concentration of NP peptide. Again, both pD^b/D^dwt and pD^b/D^dLys supported clone 3/4 binding and degranulation, with pD^b/D^dwt supporting a 3-fold greater maximal CTL binding (Fig. 4-6 A) and triggering a 5-fold stronger maximal degranulation response (Fig. 4-6 B) than pD^b/D^dLys. CTL adhesion (Fig 4-6 A) and degranulation response (Fig. 4-6 B) was density-dependent for pD^b/D^dwt and pD^b/D^dLys. When a saturating amount of peptide was used to pulse both the pD^b/D^dwt and pD^b/D^dLys at or below saturating densities (0.025 µg/well or less), 8 to 16 fold more pD^b/D^dLys molecules were required to reach the same level of CTL binding to pD^b/D^dwt pulsed with peptide (Fig. 4-6 A). About a 10-fold higher density of pD^b/D^dLys was needed to induce a similar level of clone 3/4 SE release as needed for pD^b/D^dwt with saturating peptide addition (Fig. 4-6 B). An ELISA of immobilized pD^b/D^dwt and pD^b/D^dLys was done in parallel using D^b-specific mAb, B22.249 (Fig. 4-6 C). Both pD^b/D^dwt and pD^b/D^dLys showed almost identical reactivity, confirming that at the same input of purified class I protein, the immunoreactive D^b epitopes for both pD^b/D^dwt and pD^b/D^dLys were at comparable density. In summary, these results indicate that the Glu227->Lys mutation in the $\alpha 3$ domain of class I raises the threshold of class I density required to trigger Ag-specific CTL binding and responses several fold.

and substantially lowers the maximal response achieved. This provides the first direct quantitative comparison of the ability of the wild type and $\alpha3$ mutant class I molecules to serve as Ag presenting molecules since it excludes the potential participation of both defined and undefined non-class I accessory molecule interactions.

CD8 dependency of CTL binding and degranulation to pD^b/D^{d}wt and pD^b/D^{d}Lys pulsed with NP peptide Ag. The mutation of Glu227->Lys is believed to abrogate CD8 interaction with MHC class I (Potter et al., 1989; Connolly et al., 1990; Salter et al., 1990). However, we noticed that there is some, albeit low, residual CTL adhesion and response to pD^b/D^{d}Lys pulsed with NP peptide (Fig. 4-5 and 4-6). We next compared the CD8 dependency of the CTL adhesion and degranulation response to pD^b/D^{d}wt and pD^b/D^{d}Lys pulsed with peptide. Several anti-CD8 mAbs, including 2.43 and YTS169.4, were employed to test the CD8 dependency of clone 3/4 binding and degranulation response to pD^b/D^{d}wt and pD^b/D^{d}Lys pulsed with NP peptide (Fig. 4-7). Clone 3/4 adhesion (Fig. 4-7 A and B) and SE release (Fig. 4-7 C and D) to both NP-pulsed pD^b/D^{d}wt and pD^b/D^{d}Lys were significantly inhibited by anti-CD8 mAb, 2.43 and YTS169.4. Anti-CD8 mAbs blocked 70 - 90% of clone 3/4 binding and SE release triggered by pD^b/D^{d}wt (Fig. 4-7 A and C), and completely for pD^b/D^{d}Lys pulsed with NP peptide (Fig. 4-7 B and D). This suggests that not only the pD^b/D^{d}wt, but also the low level pD^b/D^{d}Lys-triggered responses are CD8-dependent, thus the pD^b/D^{d}Lys may still interact to some extent with CD8 molecules despite the Glu 227-> Lys substitution in the $\alpha3$ domain. The degree of anti-CD8 inhibition was greater in the case of the $\alpha3$ mutated class I-peptide complexes than the wild type. This could be a result of CTL CD8 binding avidity being lower for the $\alpha3$ mutated class I, and hence is more easily blocked by anti-

CD8 mAbs. We do not believe that anti-CD8 mAb inhibition observed here is due to a "negative signal" (Julius et al., 1993) when CD8 on the CTL is bound by anti-CD8 mAbs, since the same CD8-specific antibodies used in these experiments do not interfere with immobilized anti-TCR antibody triggered CTL binding and SE release (O'Rourke et al., 1990).

Both the α3 domain wild type and mutant class I molecules serve as effective ligands for TCR-triggered CD8 adhesion and CTL response. CD8 can function both as a "coreceptor" as well as an "accessory molecule" during CTL recognition (O'Rourke and Mescher, 1993; Julius et al., 1993). Evidence suggests that when TCR is triggered, signals are generated which activate CD8 to a state of higher avidity, and consequently, the "activated", avidity-enhanced CD8 binds MHC class I molecules and facilitates CTL activation (O'Rourke et al., 1990; O'Rourke and Mescher, 1993; Kane and Mescher, 1993; Takeshita et al., 1993; Luescher et al., 1995). For instance, soluble Ab to TCR alone is not a sufficient stimulus for CTL degranulation, however it can trigger avidity-enhanced CD8 binding to non-Ag class I and cosignaling function resulting in CTL degranulation (O'Rourke et al., 1990; O'Rourke and Mescher, 1993; Kane and Mescher, 1993). As shown above, the D^b/D^dLys mutant class I could not effectively activate CTL. One possibility that could contribute to this result might be that the D^b/D^dLys molecule does not serve as an effective ligand for activated CD8 as compared to D^b/D^dwt molecules. To test this possibility, we stimulated CTL clone cells with soluble anti-TCR mAb, and determined whether CTL bind to immobilized pD^b/D^dwt and pD^b/D^dLys molecules (O'Rourke et al., 1990; O'Rourke and Mescher, 1993; Kane and Mescher, 1993). The K^b-specific clone 11 CTL were treated with soluble anti-TCRαβ mAb, H57.597, and incubated in wells coated with varying densities of pD^b/D^dwt and pD^b/D^dLys. The extent of

TCR-triggered binding was determined (Fig. 4-8 A). Clone 11 triggered by fluid-phase anti-TCR$\alpha\beta$ bound substantially to both pD^b/D^{d}wt and pD^b/D^{d}Lys in a density-dependent manner (Fig. 4-8 A), but not to purified murine class II molecules (I-E^d) immobilized at the same densities (Fig. 4-8 A). We also compared the ability of the pD^b/D^{d}wt and pD^b/D^{d}Lys molecules to provide CD8-dependent cosignals for CTL degranulation in conjunction with soluble anti-TCR antibody. Both pD^b/D^{d}wt and pD^b/D^{d}Lys were found to efficiently activate CTL degranulation response in conjunction with soluble anti-TCR mAb in a situation where soluble anti-TCR is insufficient to stimulate degranulation (Fig. 4-8 B). The soluble anti-TCR$\alpha\beta$ triggered CTL binding and degranulation to pD^b/D^{d}Lys is comparable to or slightly lower (70 - 90%) than that of pD^b/D^{d}wt throughout the immobilized class I MHC density curves (Fig. 4-8 A and B, data not shown). Both binding and SE release of clone 11 in response to either immobilized pD^b/D^{d}wt or pD^b/D^{d}Lys was blockable by an anti-CD8 mAb, 2.43 (Fig. 4-8 A and B), indicating that soluble TCR mAb triggered CTL binding to the immobilized class I molecules and SE release observed is mediated by CD8.

We have demonstrated previously that a suboptimal CTL adhesion and response to specific Ag class I can be augmented when non-Ag class I is coimmobilized (O'Rourke and Mescher, 1993; Shen and Kane, 1995). The augmentation of adhesion and degranulation by the non-Ag class I was shown to be mediated by CD8 (O'Rourke and Mescher, 1993; Shen and Kane, 1995). We examined whether pD^b/D^{d}wt or pD^b/D^{d}Lys can serve as non-Ag CD8 accessory ligands to enhance CTL binding and degranulation. Both pD^b/D^{d}wt and pD^b/D^{d}Lys were titrated and coimmobilized with a suboptimal density of purified K^b alloantigen on the wells. The binding and SE release of the K^b allo-specific clone 11 in response to suboptimal K^b and coimmobilized pD^b/D^{d}wt or pD^b/D^{d}Lys were determined (Fig. 4-9). Augmentation of clone 11 binding (Fig.

4-9 A) and triggering of degranulation (Fig. 4-9 B) facilitated by the coimmobilized pD^b/D^dwt or pD^b/D^dLys was to very similar levels throughout the class I density curves. As expected, the enhanced binding and augmented SE release facilitated by both pD^b/D^dwt and pD^b/D^dLys were mediated by CD8, as anti-CD8 mAbs significantly blocked clone 11 binding and response (Fig. 4-9 A and B).

We also tested whether the chimeric K^b with the wild type or mutant $\alpha3$ domain are effective ligands for activated CD8 as well. A suboptimal density of D^b-NP peptide complexes formed on the plastic did not trigger the D^b-restricted, NP-specific CTL clone 3/4 binding (Fig. 4-10 A) or SE release. However, when the pK^b/D^dwt or pK^b/D^dLys molecules were coimmobilized with a suboptimal density of D^b-NP complexes, they both effectively enhanced clone 3/4 binding (Fig. 4-10 A) and SE release (Fig. 4-10 B) to similar extents, which were both significantly inhibited by anti CD8 mAbs (Fig. 4-10). These results indicate that both the pK^b/D^dwt or pK^b/D^dLys serve as efficient avidity-enhanced CD8 ligands to enhance CTL binding and degranulation (Fig. 4-10). In addition, when soluble anti-TCR mAb was used instead of D^b-NP complexes as a suboptimal TCR stimulation, both the immobilized pK^b/D^dwt and pK^b/D^dLys functioned effectively to enhance CTL adhesion and degranulation.

In summary, these results show that the Glu227->Lys substitution in the $\alpha3$ domain of class I molecules does not abrogate the ability of class I to serve as a ligand for activated CD8 accessory interactions. This indicates that the Glu227 in murine class I molecules is not absolutely required for TCR-triggered CD8 binding. Since the class I $\alpha3$ domain mutants can still effectively serve as avidity-enhanced CD8 ligands, the poor ability of pD^b/D^dLys to trigger CTL adhesion and degranulation response is not explained by an inability to bind to CD8 *per se*.

The α3 Glu227->Lys substituted H-2 class I-peptide complexes are defective in initiating TCR signals for avidity-enhanced CD8 binding to non-Ag class I and CTL response. Since the D^b/D^dLys molecules can serve as effective non-Ag class I ligands for TCR-activated, avidity-enhanced CD8 (Fig. 4-8, 4-9 and 4-10), we investigated whether D^b/D^dLys-peptide complexes are defective in initiating TCR signals for avidity-enhanced CD8-dependent CTL adhesion and response. This is a direct test for whether class I molecules are effective ligands for CD8 coreceptors, as CD8 coreceptor function is essential in facilitating TCR/class I interactions for CD8-dependent CTL activation (Salter et al., 1990; Julius et al., 1993; Miceli and Parnes, 1993). It has been shown that a suboptimal density of class I or soluble anti-TCR mAb, which is not sufficient to fully activate CTL, can initiate TCR signalling which in turn enhances CD8 binding to non-Ag MHC class I and augments CTL adhesion and responses (O'Rourke et al., 1990; Kane and Mescher, 1993; Takeshita et al., 1993; Luescher et al., 1995). To test their effectiveness in initiating early TCR-dependent events in T cell activation, both the pD^b/D^dwt and pD^b/D^dLys were titrated and coated on the plastic with or without coimmobilization of a non-Ag class I molecule, K^k. After pulsing the immobilized class I with NP peptide, clone 3/4 CTL cells were added to the wells and their enhanced binding and SE release to coimmobilized non-Ag class I were determined (Fig. 4-11). Coimmobilized K^k greatly augmented clone 3/4 adhesion (Fig. 4-11 A) and SE release (Fig. 4-11 B) in response to pD^b/D^dwt-NP complexes. This effect was observed at the pD^b/D^dwt input as low as 0.0008 μg/well for enhanced binding (Fig. 4-11 A), and 0.006 μg/well for augmented degranulation (Fig. 4-11 B), while at these concentrations, pD^b/D^dwt alone does not trigger CTL adhesion or degranulation response. In contrast, over a range up to 0.05 μg/well input (approaching immobilization saturation),

pD^b/D^dLys did not trigger CTL for enhanced binding or SE release when coimmobilized with K^k (Fig. 4-11 A and B). These results indicate that the α3 domain mutation in MHC class I molecules likely affects initial low avidity or basal CD8 binding to class I-peptide Ag complexes which interferes with its CD8 coreceptor function, but not subsequent avidity-enhanced CD8 binding and accessory function.

D. Conclusions

Using immobilized purified chimeric class I molecules consisting of $D^b\alpha1\alpha2$ or $K^b\alpha1\alpha2$ and $D^d\alpha3$ domains, in which the $D^d\alpha3$ domain is either the wild type or the mutant with a Glu227 ->Lys substitution, we assessed the potential role of Glu227 in the α3 domain acidic loop of class I in CD8 coreceptor and TCR-triggered avidity-enhanced CD8 accessory functions of cloned CTL. It is directly demonstrated that the mutant class I effectively serves as an activated CD8 accessory ligand, indicating that Glu227 is not required for this type of CD8 interaction. In contrast, the purified mutant class I molecules were found to be defective in triggering CTL responses due to an inability to effectively co-engage TCR and CD8 to initiate TCR-dependent activation events.

E. Discussion

Quantitative differences of MHC class I with wild type or mutated α3 domains in triggering CTL. MHC class I molecules bearing a Glu227->Lys α3 domain mutation has been shown to substantially diminish or abrogate CD8-dependent CTL lysis (This study and Potter et al., 1987 & 1989; Connolly et al., 1990; Knall et al., 1994). However, a direct comparison between the class I α3 wild type and those with mutations in the $\alpha3$ domain in mediating Ag specific, CD8-dependent CTL adhesion and responses has not been achieved before. This is mainly because of the participation of other molecules involved in CTL recognition and activation when cells, instead of purified class I molecules, are used. In the present study, quantitative differences of MHC class I with wild type or mutated $\alpha3$ domains in triggering CTL was explored using purified MHC molecules on solid phase, where the density of class I and the number of peptide antigen-class I complexes were varied in a controlled manner. The number of peptide-class I complexes formed on solid phase with the immobilized wild type and mutant class I by addition of exogenous peptide antigen are likely to be very similar, if not identical, since the addition of exogenous peptide antigen induces peptide-dependent conformational epitopes to an equivalent extent on wild type and $\alpha3$ domain mutated class I molecules expressed on endogenous peptide loading defective T2 cells (Knall et al., 1994). Furthermore, the acid-eluted endogenous peptides of K^b and D^b molecules with their respective $\alpha3$ mutant counterparts appear identical, suggesting that the point mutation in the $\alpha3$ domain does not affect peptide binding (Knall et al., 1994; Dutz et al., 1995).

We found the maximal CTL adhesion and degranulation response to be at least three fold greater to immobilized purified wild type as opposed to mutant class I presenting molecules pulsed with peptide. The mutant class I molecules did not achieve greater than ten percent of input CTL binding, which was only observed at high concentrations of antigenic peptide. These results are consistent with the differences in target cell lysis and degranulation responses we found with the same CTL clone in response to wild type and mutant class I bearing target cells. In experiments not easily approached using target cells, we show that in the presence of saturating peptide antigen, it requires an 8-16 fold higher density of immobilized mutant class I molecules to achieve the same level of CTL adhesion and response as with molecules bearing a wild type α3 domain.

We show that the purified Glu227->Lys α3 mutant molecules bound with peptide antigen are very poor ligands for CTL adhesion and response, yet there is still some low level CTL reactivity to these mutant class I-antigen complexes and a direct relationship remains between CTL adhesion level and degranulation response. It is not clear how the CD8-dependent CTL clone is responding to the mutant class I molecules, however responses are only found at relatively high concentrations of peptide and this is also true for peptide-pulsed target cells (Fig. 4-1 and 4-2; Knall et al., 1994). When presented with a high density of peptide-class I complexes, TCR engagement and triggering will be less dependent on accessory molecule interactions, including CD8 (Alexander et al., 1991; Al-Ramadi et al., 1995). The CTL responses to the mutant class I complexes on cells and solid phase however were partially or completely inhibited by CD8-specific antibodies, suggesting that CD8 is still participating in mutant class I molecule recognition. The affinity of TCR for antigen can vary considerably, but is generally quite low (Corr et al., 1994). In the less complicated and more readily interpretable solid phase system, at high mutant class I-peptide antigen density, the combination of multiple low affinity TCR and residual low avidity CD8 interactions may be sufficient to support the low level binding and response observed with the mutant class I presenting molecules and peptide. Alternatively or additionally, at high mutant class I-antigen complex density there may be some minimal TCR dependent signalling resulting in a small degree of avidity enhancement of CD8 binding, but if this occurs it is inefficient, since we only observe a low level of CTL binding and response.

The α3 domain Glu227->Lys substitution disrupts class I interaction with the CD8 coreceptor. Several amino acid substitutions in the acidic loop of the conserved α3 domain of class I disrupt CD8-dependent CTL antigen recognition (Potter et al., 1987 & 1989; Connolly et al., 1990; Salter et al., 1989 & 1990; Sakimata et al., 1993). This is likely to be due to interference with CD8 coreceptor function since the mutagenesis of the antigen presenting class I, the ligand for the TCR, blocks response despite the presence of non-Ag class I on the same cell membrane, which in theory could serve as accessory ligands for CD8. More direct evidence for CD8 serving a coreceptor adhesive role has been provided recently by Luescher et al. who examined CTL interactions with soluble wild type and α3 mutant K^d class I molecules (Luescher et al., 1995). Using photoaffinity labelling techniques, they directly demonstrated that CD8 can serve as a coreceptor by significantly strengthening cell surface TCR binding to soluble K^d on an allo-specific CTL clone and this interaction was shown to be inhibited by a Lys substitution for the Asp227 in K^d. However, there was no analysis of functional responses possible in this system to determine the relationship of binding to response, since class I in soluble form is not a stimulus for T cell responses. Our studies with solid phase class I provide a quantitative comparison of class I molecules bearing wild type or the α3 domain mutation for CTL adhesion and response. When incubated with peptide antigen, the mutant class I molecules were unable to trigger TCR-dependent signals necessary for the induction of avidity-enhanced CD8 binding to coimmobilized non-antigen class I. Our data are consistent with and strengthen the conclusions of previous reports suggesting that the Glu227 mutation disrupts CD8 coreceptor interactions, as our results reveal that CD8 coreceptor engagement with peptide antigen class I complexes is necessary for the TCR-dependent expression of avidity-enhanced CD8 binding to non-Ag class I and CD8 accessory function by CTL.

Expression of avidity-enhanced CD8 is dependent on initial TCR-class I interaction with the participation of intact CD8 coreceptor function. Binding and response to class I bearing cells or surfaces by CTL appears to be a dynamic multistep process controlled by the TCR (O'Rourke and Mescher, 1993). For example, no detectable CTL binding is observed to immobilized non-Ag class I in the absence of a TCR stimulus (O'Rourke et al., 1990; Kane and Mescher, 1993), thus CD8 interaction with class I is likely to be minimal in the absence of TCR triggering. However, if the TCR is engaged with immobilized specific antigenic class I, high avidity CD8 accessory interactions with coimmobilized non-Ag class I is observed and this results in the delivery of additional signals leading to phosphatidylinositol hydrolysis and degranulation of CTL (O'Rourke et al., 1990; Kane and Mescher, 1993; O'Rourke and Mescher, 1994). That this process is likely to be sequential is supported by the observation that pretreatment of CTL with low concentrations of soluble antibodies to the TCR used to mimic antigen recognition, triggers subsequent high avidity CD8 binding to class I (O'Rourke et al., 1990; Kane and Mescher, 1993). The CD8 avidity enhancement is blocked by inhibitors of tyrosine phosphorylation suggesting that this process is dependent on intracellular signalling (O'Rourke et al., 1990; O'Rourke and Mescher, 1994). Since the $\alpha3$ domain mutant class I is defective in supporting appropriate triggering through TCR for CD8 binding in the presence of an excess of coimmobilized non-Ag class I (Fig. 4-11), our results suggest a sequential model of CD8 receptor functions. In this model, the Glu227 of H-2 class I is essential for early low avidity CD8 coreceptor function in stabilizing TCR engagement with class I and facilitating initial TCR-activation signals, but not for subsequent avidity-enhanced CD8 accessory molecule recognition and function which may involve additional binding sites on class I molecules. We do not exclude the possibility that when CD8 undergoes avidity enhancement it may also augment CD8 coreceptor function, but the expression of activated CD8 binding regardless of whether the class I ligand is presenting the peptide antigen or not, would still be dependent on initial low avidity CD8 interactions.

It has been difficult to determine the relative contributions of CD8 coreceptor and accessory functions to CTL activation. However, since mutagenesis of the α3 domain of the specific Ag presenting class I molecule (potential CD8 coreceptor ligand) can ablate CD8-dependent CTL reactivity even in the presence of endogenous non-Ag class I (potential CD8 accessory ligand), it has been suggested that CD8 functions much more efficiently as a coreceptor than an accessory molecule (Salter et al., 1990; Knall et al., 1994). Results in the present report indicate that this simple interpretation can be significantly revised and clarified. Our data support the conclusion that the induction and expression of avidity-enhanced CD8 accessory function is simply *dependent* on an initial CD8 coreceptor priming function and CD8 accessory interactions can be critical for CTL responsiveness at low antigen density. Thus both CD8 coreceptor and accessory functions are important for CTL activation. Without the initiating TCR-dependent CD8 coreceptor activity which is prevented or greatly diminished by the Glu227->Lys mutation, however, no avidity enhancement of CD8 occurs in response to α3 mutant class I-peptide antigen complexes and no subsequent CD8 accessory function can be observed. It has been a conceptual problem that why little or no response is observed when peptide antigen is presented by the α3 domain mutant class I, despite the presence of non-Ag class I that in principle might be expected to compensate as CD8 accessory ligands for deficiencies in the α3 mutated class I presenting molecules (Connolly et al., 1990; Salter et al., 1990; Ingold et al., 1991; Knall et al., 1994). Here, our results provide an explanation for this apparent paradox.

It is interesting to note that intact CD8 coreceptor function is not only critical for the subsequent avidity-enhanced CD8 accessory function, but may also be important for avidity enhancement of other adhesion molecules. Ybarrondo et al. (1994) has shown that a suboptimal density of antigenic MHC class I molecules triggered avidity-enhanced LFA-1 binding to immobilized ICAM-1 is subject to anti-CD8 mAb blocking, suggesting that CD8 coreceptor function is involved in the "activation" of LFA-1. This result suggest that CD8 coreceptor function may have a more general role in CTL activation by upregulating the avidity of a number of adhesion receptors in addition to CD8.

Avidity-enhanced CD8 binding of MHC class I. Surprisingly, we observed CTL avidity-enhanced CD8 binding induced by soluble anti-TCR mAb or alloantigen not only to class I bearing a wild type $\alpha3$ domain but also to class I $\alpha3$ mutants which lack a critical CD8 binding site. Furthermore, additional signaling associated with avidity-enhanced CD8 binding was only modestly reduced or undiminished with the $\alpha3$ domain mutant class I relative to wild type molecules in the two experimental systems employed. These clearly demonstrate that the $\alpha3$ domain mutant class I molecule is still an effective ligand for avidity-enhanced CD8 binding, however it is unclear at present how CD8 binds the mutant molecules. This is hampered in part by the lack of understanding regarding how the avidity of CD8 is modulated by TCR triggering. We propose several possible scenarios for how activated CD8 might interact with the $\alpha3$ mutated MHC class I molecules.

1. Activated CD8 still interacts with MHC class I $\alpha3$ domain

It is clear from cell transfection and adhesion assays that the conserved $\alpha3$ domain residue 222-229 acidic loop that includes Glu/Asp227 is a binding site on class I for CD8α homodimers when CD8α is overexpressed (Salter et al., 1990).

Independent confirmation of an α3 binding site for CD8α was provided by Fayen et al. who showed that the human CD8α IgV domain and the α3 domain of HLA-A2.1 can interact in soluble and immobilized forms and thus these domains of CD8 and class I respectively, are sufficient for binding *in vitro* (Fayen et al., 1995). It is possible that avidity-enhanced CD8 binding to mutant class I is still to the acidic loop of class I α3 domain, however its binding may be less dependent than CD8 in its low avidity state on the presence of Glu227. It has been reported previously that avidity-enhanced CD8 binding is diminished by agents that inhibit cytoskeletal modifications (O'Rourke et al., 1991). A consequence of cytoskeletal changes in response to TCR triggering may be an increase in cell spreading resulting in an increased surface area of contact. By increasing the area of contact, CTL would increase the quantity of CD8 that might engage class I on a target cell surface or on solid phase and the increased number of interactions of CD8 with class I between the two surfaces may be sufficient to support stable binding. A second consequence of cytoskeletal rearrangements induced by TCR triggering may be CD8 microclustering as found with the C3bi receptor (Detmers et al., 1987), increasing the multivalency of CD8 and in turn increase the avidity but not necessarily the affinity of CD8 for MHC class I. In both of these scenarios, avidity increases between the CD8 and class I bearing surfaces may compensate for reductions in CD8 affinity from the Glu227 ->Lys mutation without a change in the overall CD8 binding site on class I.

2. Activated CD8 binds additional sites on class I

It has recently been reported by Sun et al. using transfection and overexpression systems that substitutions in the α2 domain of HLA class I can disrupt cell-cell adhesion mediated by human CD8αα , suggesting that CD8 may have additional contact or binding sites on class I outside the α3 domain (Sun et al., 1995). Furthermore, recent studies employing exon shuffling of domains between human and mouse class I genes, have provided evidence to suggest that the $\alpha2$ domain is likely to influence CD8 interactions with MHC class I in T cell development and mature $CD8^+$ T cell reactivity (LeFace et al., 1995; Newberg et al., 1996). Although additional CD8 binding sites on class I may be involved in basal or low avidity CD8 binding, CD8 interaction with these additional sites may be favored upon TCR triggering and support activated CD8 binding despite the Glu227->Lys mutation. It may be informative to determine whether activated CD8 binding and accessory function is abrogated or sustained if corresponding critical residues in the acidic loop and $\alpha2$ domain of class I that disrupt $CD8\alpha\alpha$ binding are both mutated. Whether other sites on class I may still be involved in TCR-activated, avidity-enhanced CD8 binding remains to be determined. Since the activated CD8 binding to MHC class I does not involve the participation of TCR, it is conceivable that class I is more accessible to CD8 interaction under this circumstance. It is not surprising if activated CD8 accessory type binding has a less stringent structural requirement for MHC class I.

3. Activated CD8 undergoes conformational changes

The preceding discussion has not considered structural changes of CD8 that may result from TCR triggering. LFA-1 molecules on T cells can be activated to a much higher affinity state, presumably through conformational changes (Cabanas and Hogg, 1993; Lollo, et al., 1993). It is conceivable that the CD8 heterodimer can also undergo a conformational change resulting from TCR-triggered intracellular signals, which enhance CD8 affinity for the $\alpha3$ acidic loop or alternatively allows CD8 to bind additional sites on class I. This may provide an explanation for our observation of activated CD8 adhesion to the mutant molecules. In this context, Meyerson et al. have found that the presence of the membrane proximal connecting peptide or stalk of CD8 reduces N terminal $CD8\alpha$ IgV domain binding to the $\alpha3$ domain and the binding of purified $CD8\alpha$ in soluble form to class I is temperature sensitive (Meyerson et al., 1996). These results could suggest that a specific or preferred conformation of CD8 or class I may be necessary for binding. Perhaps TCR triggered changes in CTL lead to the induction or stabilization of appropriate CD8 conformation(s) for class I binding.

4. Participation of CD8 β chains

In contrast to the report of Meyerson et al. (1996) and others involving CD8αα homodimers, our study examines class I adhesion by CD8αβ heterodimers which are typically expressed on mature murine $CD8^+$ T cells. Expression or lack thereof, of the CD8 β chain has been shown to substantially influence T cell thymic maturation (Nakayama et al., 1994; Fung-Leung et al., 1994; Crook and Littman; 1994) and CD8 β undergoes structural changes depending on T cell differentiation and activation state (Casabo et al., 1994). It is less clear whether and how the CD8 β chain might directly participate in mature T cell recognition of class I (Letourneur et al., 1990; Wheeler et al., 1992; Karaki et al., 1992). The results presented in Chapter III clearly demonstrate that CD8 β chain is not required for activated CD8 binding to class I, however, it remains to be determined whether the CD8 β chain may influence or possibly regulate basal or avidity-enhanced CD8 binding to class I.

Table 4-1. FACS analysis of M12.D^b/D^{d}wt and M12.D^b/D^{d}Lys transfectant cells [1]

1st mAbs[2]	Specificity	M12.D^b/D^{d}wt	M12.D^b/D^{d}Lys
Control		3.79[3]	3.22
B22.249	D^b $\alpha1\alpha2$	93.90	90.58
34-5-8s	D^d $\alpha1\alpha2$	47.70	41.42
34-2-12s	D^d $\alpha3$	50.94	40.32
Y3	K^b $\alpha1\alpha2$	5.38	5.57

[1] FACS analysis of M12.D^b/D^{d}wt and M12.D^b/D^{d}Lys transfectant cells determined with various mAbs.

[2] First antibody used in FACS analysis: purified B22.249 and Y3 were used at 1.5 µg/ml; 34-5-8s and 34-2-12s were used as culture supernatants in 10% FCS-RPMI at 1:4 dilution.

[3] Results are expressed as mean fluorescence intensity.

Table 4-2. ELISA Reactivity of pD^b/D^dwt and pD^b/D^dLys [1]

mAbs[2]	Specificity	pD^b/D^dwt	pD^b/D^dLys
B22.249	D^b α1α2	1.062 ± 0.052[3]	1.092 ± 0.048
34-5-8s	D^d α1α2	0.003 ± 0.001	0.013 ± 0.002
34-2-12s	D^d α3	0.347 ± 0.032	0.009 ± 0.002
Y3	K^b α1α2	0.005 ± 0.001	0.017 ± 0.001

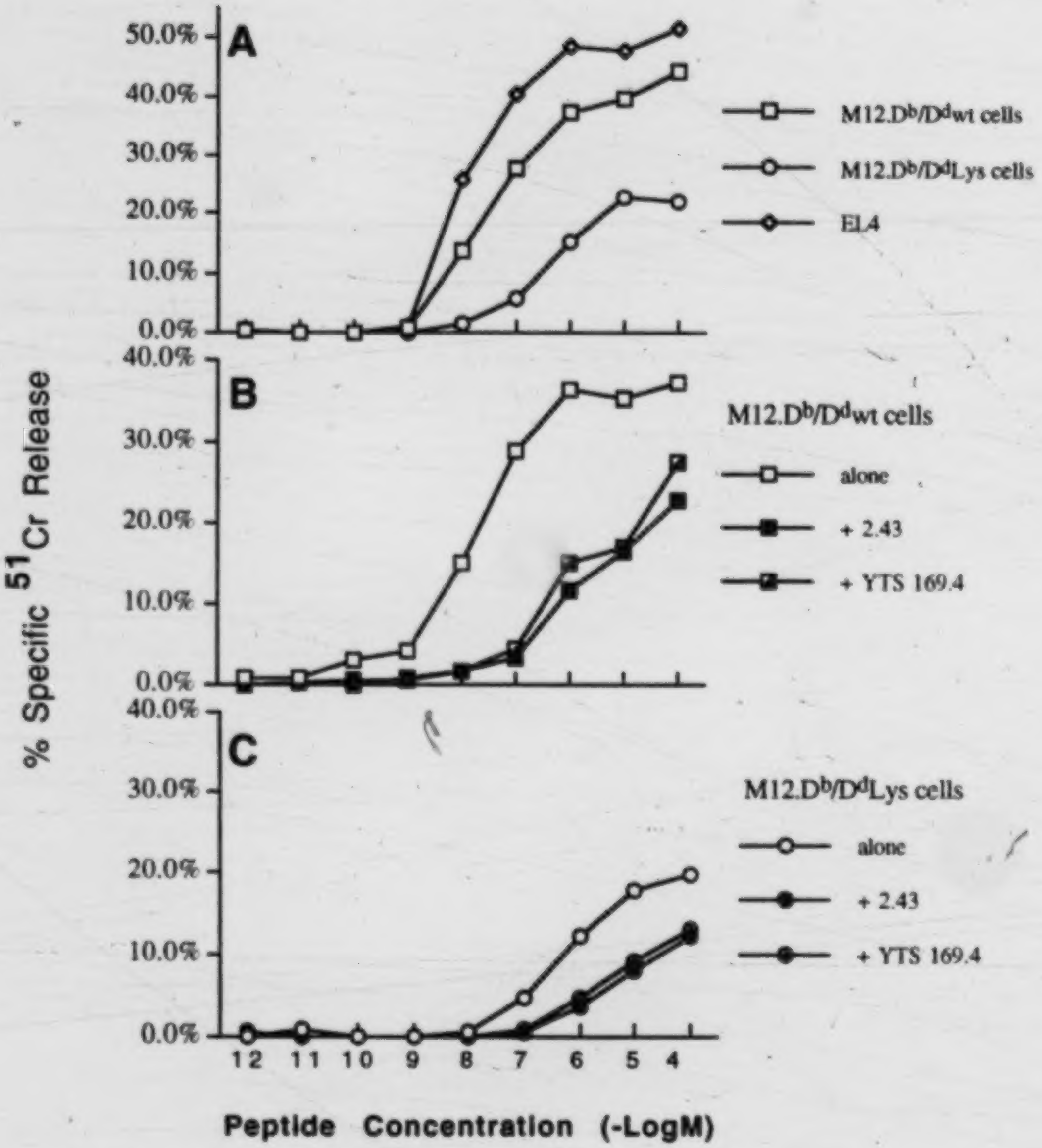
[1] ELISA reactivity of pD^b/D^dwt and pD^b/D^dLys determined with various mAbs.

[2] First antibody used in solid-phase ELISA assay: purified B22.249 and Y3 were diluted into 2% FCS-PBS and used at 2.5 μg/ml; 34-5-8s and 34-2-12s were used as culture supernatants in 10% FCS-RPMI at 1:4 dilution.

[3] Results are expressed as mean OD_{490nm} ± SD of triplicate wells.

Figure 4-1. Antigen-specific, CD8$^+$ CTL clone lysis of target cells bearing MHC class I molecules with wild type or mutant $\alpha3$ domains. (A) Lysis of M12.D^b/D^{d}wt, M12.D^b/D^{d}Lys transfectant cells and EL4 cells by an influenza NP-specific CTL clone, clone 3/4. The ^{51}Cr-labeled target cells were pulsed with the influenza NP peptide (Y-NP366-374) at the indicated concentrations. 1 x 10^4 targets were incubated together with clone 3/4 effector cells at a 5:1 E:T ratio at 37°C for 5 h. In separate experiments, effects of anti-CD8 antibodies on clone 3/4 CTL killing of M12.D^b/D^{d}wt (B) and M12.D^b/D^{d}Lys (C) cells were determined. Clone 3/4 effector cells were incubated with anti-CD8 antibodies at RT for 30 min before incubating with ^{51}Cr-labeled, NP peptide pulsed target cells. 2.43 mAb was used as 1:4 dilution of culture supernatant and purified YTS169.4 used at the concentration of 5 μg/ml. No lysis of targets was seen without NP peptide pulsing. In all the experiments, percent specific lysis is expressed as the mean of triplicate wells.

Figure 4-1


A
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
M12.Db/Ddwt cells
M12.Db/DdLys cells
EL4
B
40.0%
30.0%
20.0%
10.0%
0.0%
M12.Db/Ddwt cells
alone
+ 2.43
+ YTS 169.4
C
40.0%
30.0%
20.0%
10.0%
0.0%
M12.Db/DdLys cells
alone
+ 2.43
+ YTS 169.4
% Specific 51Cr Release
12
11
10
9
8
7
6
5
4
Peptide Concentration (-LogM)

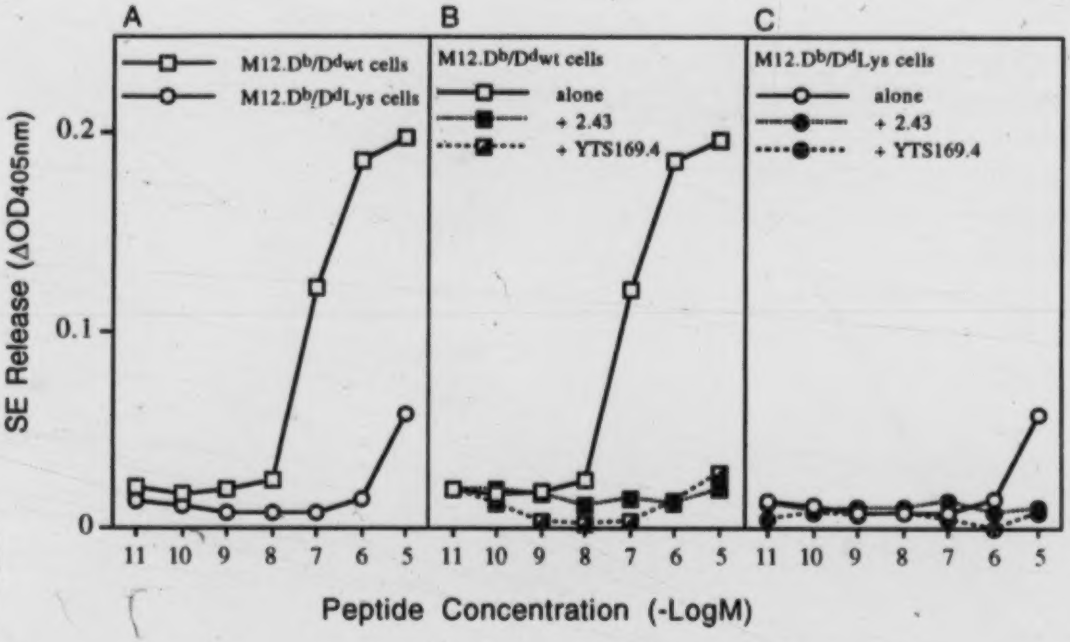


Figure 4-2. Antigen-specific CTL clone degranulation responses to cells bearing wild type or α3 domain-mutated class I molecules. (A) Degranulation of an influenza NP-specific CTL clone, 3/4, in response to M12.D^b/D^dwt and M12.D^b/D^dLys transfectants. Both transfectants were pulsed with the NP peptide at the indicated concentrations. After washing, 3 x 10^5 transfectant cells were incubated together with 1 x 10^5 clone 3/4 cells at 37°C for 4 h. Effects of anti-CD8 antibodies on clone 3/4 degranulation in response to M12.D^b/D^dwt (B) and M12.D^b/D^dLys (C) cells were also determined. Clone 3/4 cells were incubated with anti-CD8 antibodies at RT for 30 min before incubating with the peptide-pulsed transfectant cells. Anti-CD8 mAb, 2.43, was used at 1:4 dilution of culture supernatant, and YTS169.4 used at the concentration of 5 μg/ml. In all the experiments, SE release is expressed as the mean of triplicate wells.

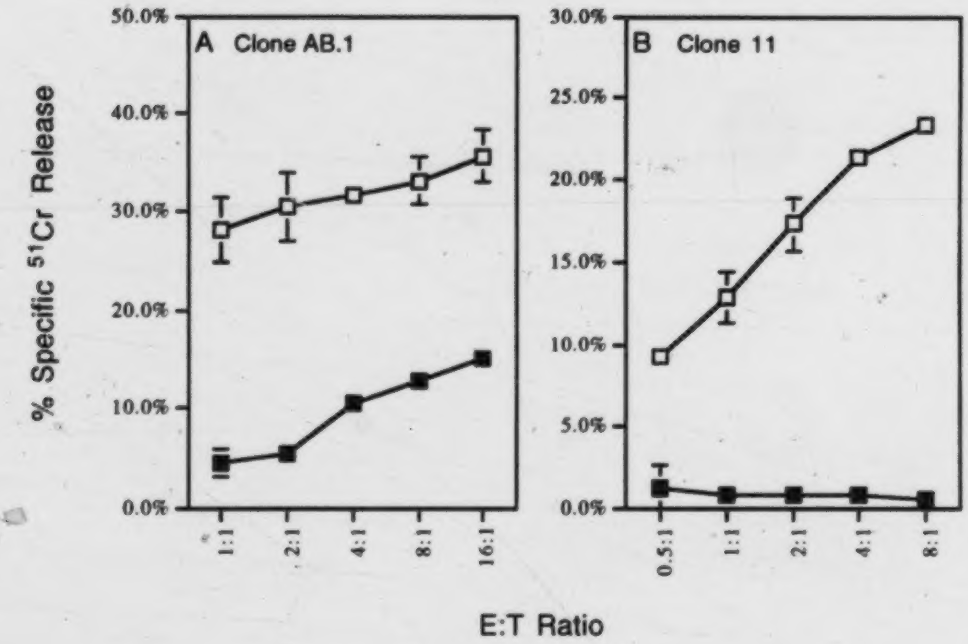


Figure 4-3. K^b allo-specific CTL lysis of M12 cells expressing wild type or $\alpha 3$ domain-mutated class I K^b molecules. Lysis of M12.K^b/D^dwt (*open squares*) and M12.K^b/D^dLys (*solid squares*) transfectant cells by K^b allo-specific CTL clones, AB.1 (A) and 11 (B) was determined by ^{51}Cr release assays. Target cells (1×10^4) were incubated with clone AB.1 or 11 effector cells at the indicated E:T ratio at 37°C for 5 h. Lysis of EL4 (H-2^b) by clone AB.1 and 11 (both at 2:1 E:T ratio) was 81% and 74%, respectively. No lysis (less than 1%) of A20 (H-2^d) or P815 (H-2^d) cells was seen. In all the experiments, spontaneous release was less than 15% of the total. Assays were carried out in triplicate. Results are expressed as mean percent specific lysis ± SD.

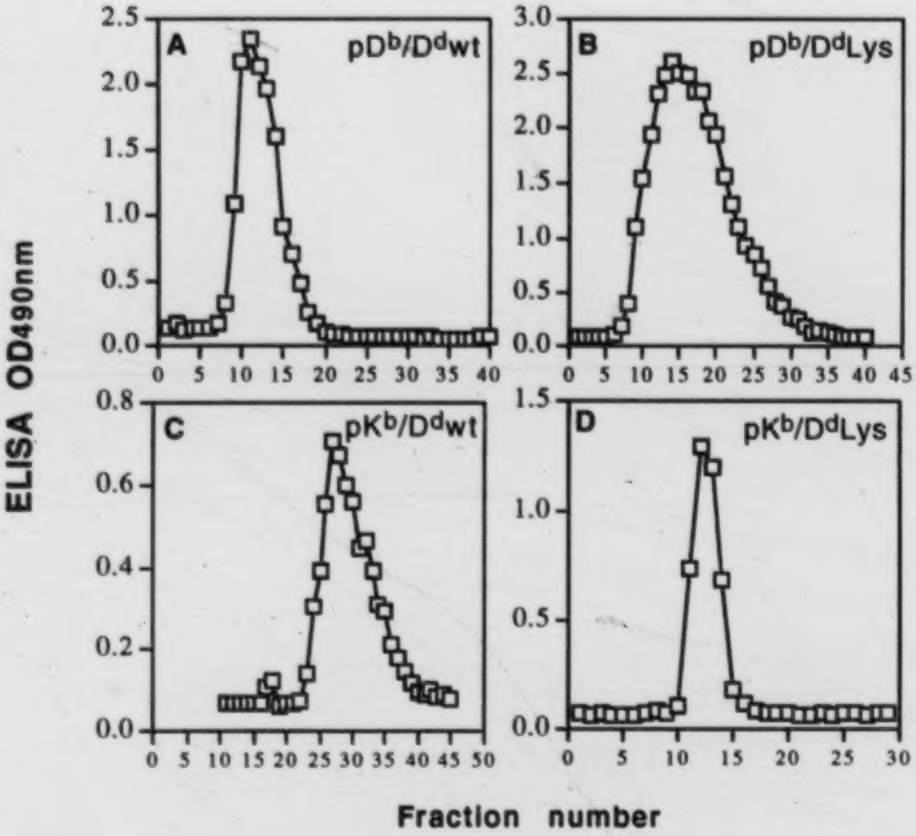


Figure 4-4. Immunoaffinity purification of chimeric class I D^b and K^b molecules. The D^b/D^dwt (A) and D^b/D^dLys (B) chimeric class I molecules were isolated from M12.D^b/D^dwt and M12.D^b/D^dLys cells respectively, by using separate B22 mAb immunoaffinity columns. The K^b/D^dwt (C) and K^b/D^dLys (D) molecules were isolated from M12.K^b/D^dwt and M12.K^b/D^dLys cells respectively, by using separateY3 immunoaffinity columns. Each eluted fraction (1:20 dilution) was assayed for mAb reactivity by solid-phase ELISA. B22.249 mAb reactivity was measured for pD^b/D^dwt (A) and pD^b/D^dLys (B) fractions, while Y3 reactivity was determined for pK^b/D^dwt (C) and pK^b/D^dLys (D) fractions. Results are expressed as an average of OD_{490nm} of duplicate wells.

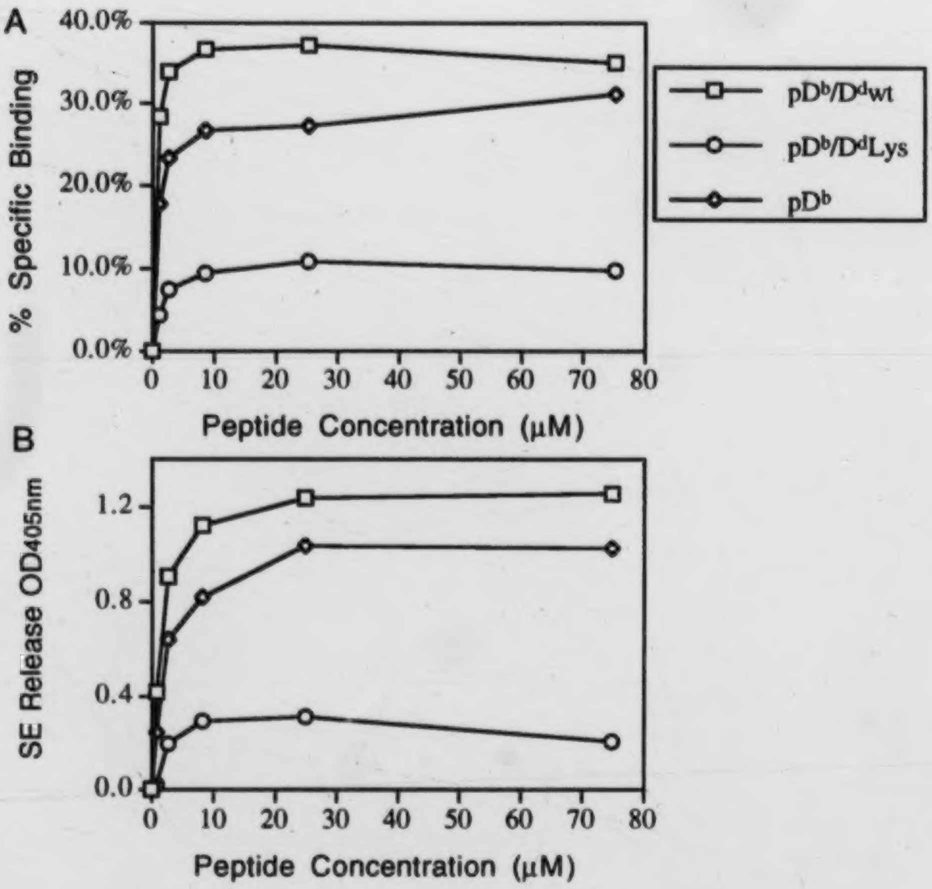


Figure 4-5. Comparison of influenza NP-specific CTL clone adhesion and SE release to immobilized pD^b/D^dwt and pD^b/D^dLys complexed with NP peptide. The pD^b/D^dwt, pD^b/D^dLys and pD^b (isolated from EL4 cells) were coated on microtitre plate wells at a density of 0.1 μg/well, and pulsed with NP peptide at the concentrations indicated. Clone 3/4 CTL were applied at 2 x 10^5 per well, and incubated at 37°C for 4 h. The CTL adhesion (A) and degranulation as measured by SE release (B) were determined simultaneously. Non-specific binding (3.4%) was subtracted. Results are expressed as the mean of triplicate wells.

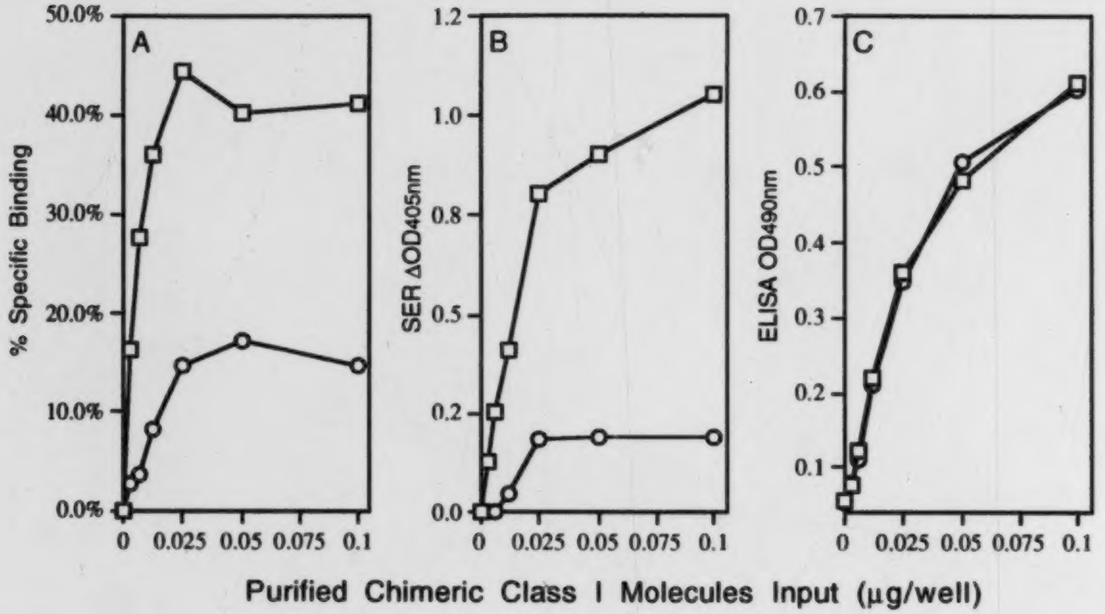


Figure 4-6. Density dependence of isolated wild type and mutated α3 domain class I for Ag-specific CTL adhesion and SE release response. The pD^b/D^{d}wt (*open squares*) and pD^b/D^{d}Lys (*open circles*) were immobilized on microtitre plate wells at the indicated density, and pulsed with NP peptide at a saturating concentration of 75 µM. NP-specific clone 3/4 CTL cells were loaded into the wells at 2 x 10^5 per well, and incubated at 37°C for 4 h. The CTL adhesion (A) and degranulation as measured by SE release (B) were done simultaneously from the same wells. Non-specific binding (4.1%) was subtracted. (C) Solid-phase ELISA of pD^b/D^{d}wt and pD^b/D^{d}Lys molecules. The same pD^b/D^{d}wt (*open squares*) and pD^b/D^{d}Lys (*open circles*) molecules used in binding (A) and SE release (B) assays were titrated and coated on the plates at the same densities. Their ELISA reactivities were determined by using a D^b-specific mAb, B22.249, and are expressed as the mean of duplicate wells.

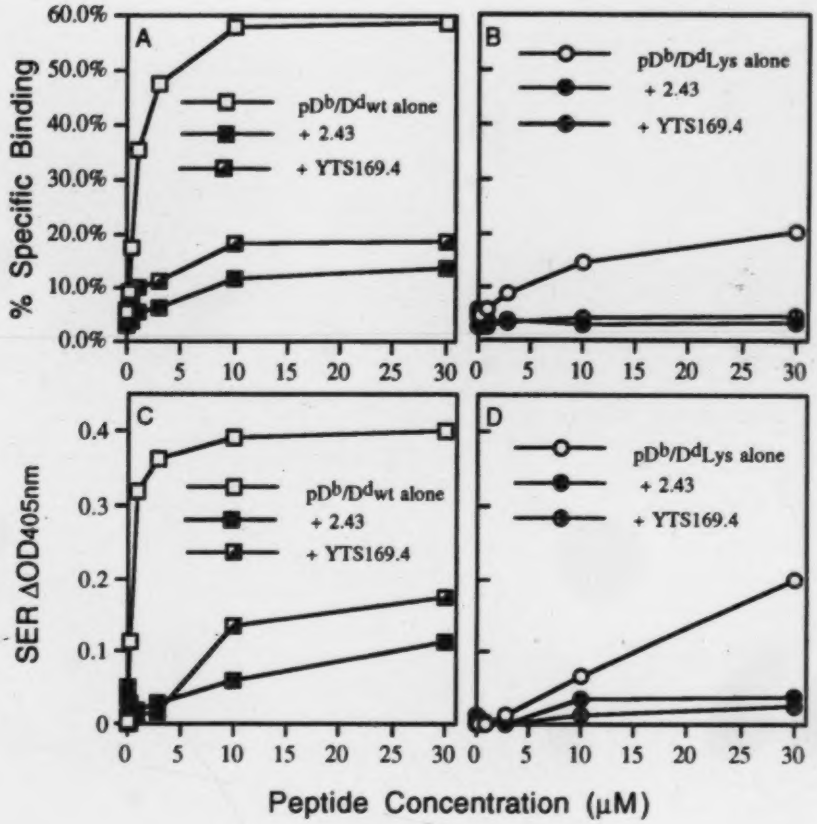


Figure 4-7. CD8-dependency of NP-specific CTL adhesion and degranulation in response to the pD^b/D^dwt- and pD^b/D^dLys-NP peptide complexes. Both pD^b/D^dwt (A and C) and pD^b/D^dLys (B and D) were immobilized on the plastic at a density of 0.1 μg/well, and pulsed with NP peptide at the concentrations shown. Clone 3/4 CTL cells were loaded into the wells at 2 x 10^5 per well, and incubated at 37°C for 4 h. The CTL adhesion (A and B) and degranulation as measured by SE release (C and D) were carried out simultaneously. Anti-CD8 mAb inhibition of both adhesion and SE release were performed as described in *Materials and Methods*. Anti-CD8 mAb, 2.43, was used at 1 : 4 dilution of culture supernatant, and YTS169.4 was used at the concentration of 5 μg/ml. All results are the mean of triplicate wells.

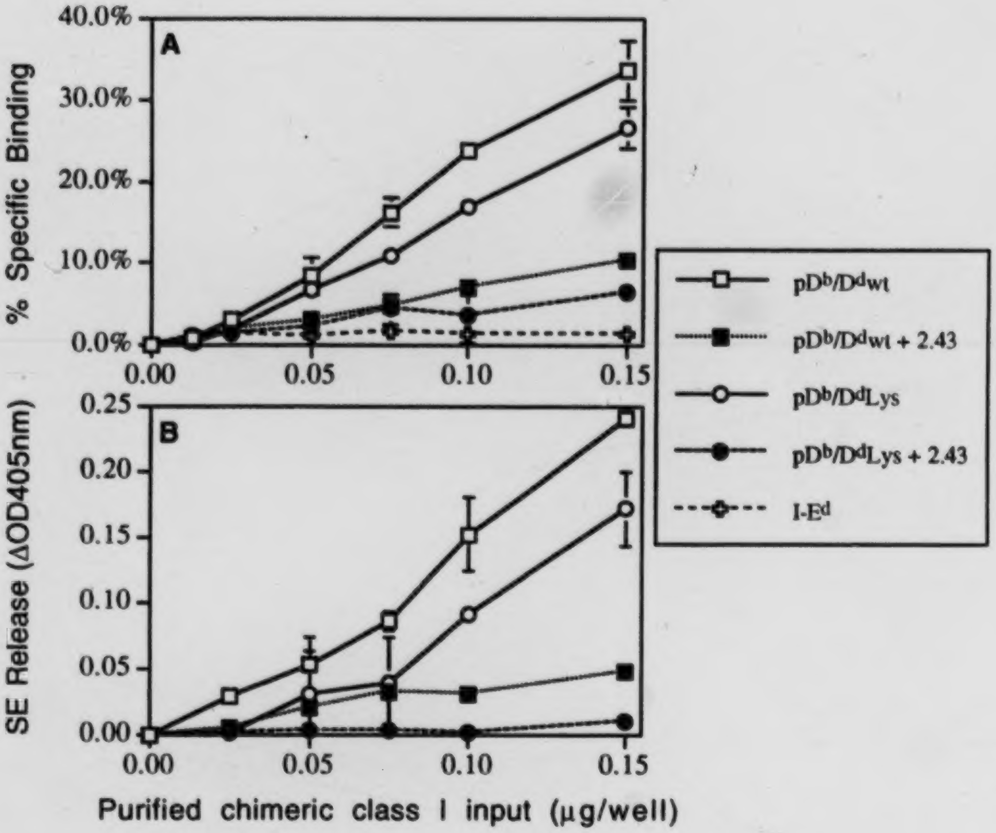


Figure 4-8. Soluble anti-TCRαβ mAb triggers CD8-dependent CTL binding and response to pD^b/D^{d}wt and the α3 domain mutant, pD^b/D^{d}Lys. The pD^b/D^{d}wt, pD^b/D^{d}Lys or I-E^d was separately immobilized on the wells at the indicated densities. The K^b alloreactive clone 11 CTL were resuspended with the anti-TCRαβ mAb, H57.597, at a concentration of 0.5 μg/ml immediately before loading into wells. For anti-CD8 mAb blocking, clone 11 cells were incubated at RT for 30 min with 2.43 mAb (1: 4 dilution of culture supernatant) before the addition of anti-TCR mAb. 1.5 x 10^5 clone 11 cells were loaded into wells and incubated at 37°C for 4 h. Clone 11 binding (A) and SE release (B) of clone 11 cells were determined. Results were expressed as the mean ± SD. Clone 11 binding to BSA was 3.9% treated as background and subtracted. Clone 11 without soluble anti-TCRαβ stimulation bound to 0.15 μg/well input of both pD^b/D^{d}wt and pD^b/D^{d}Lys to a similar background level as clone 11 binding to BSA.

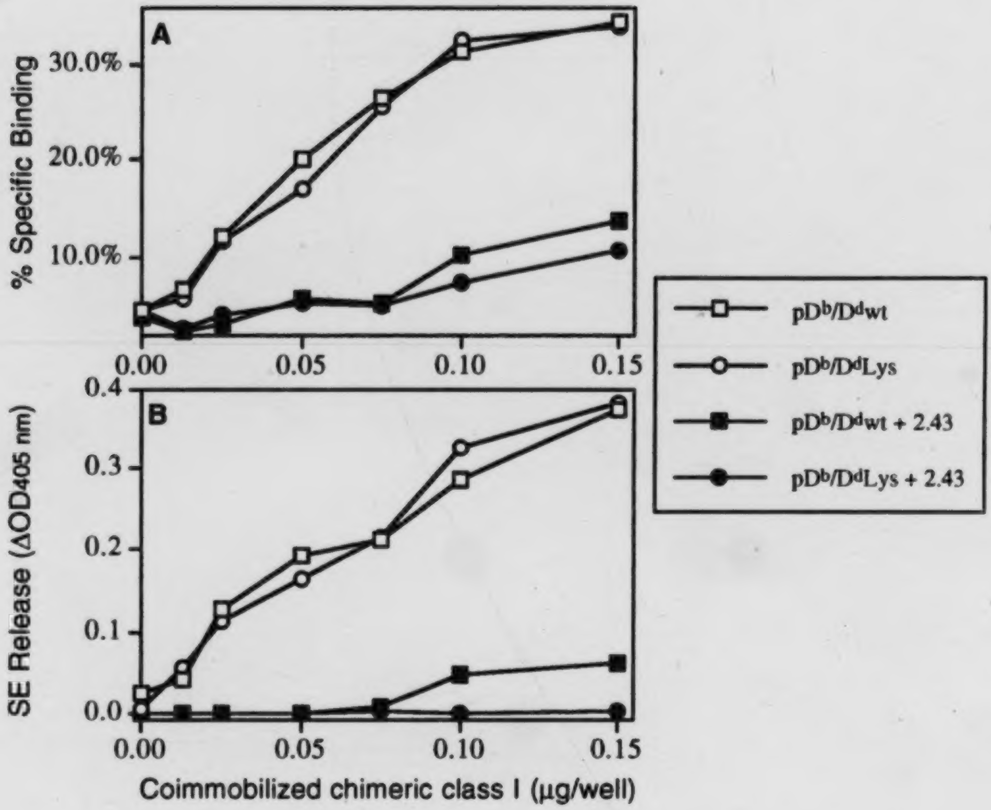
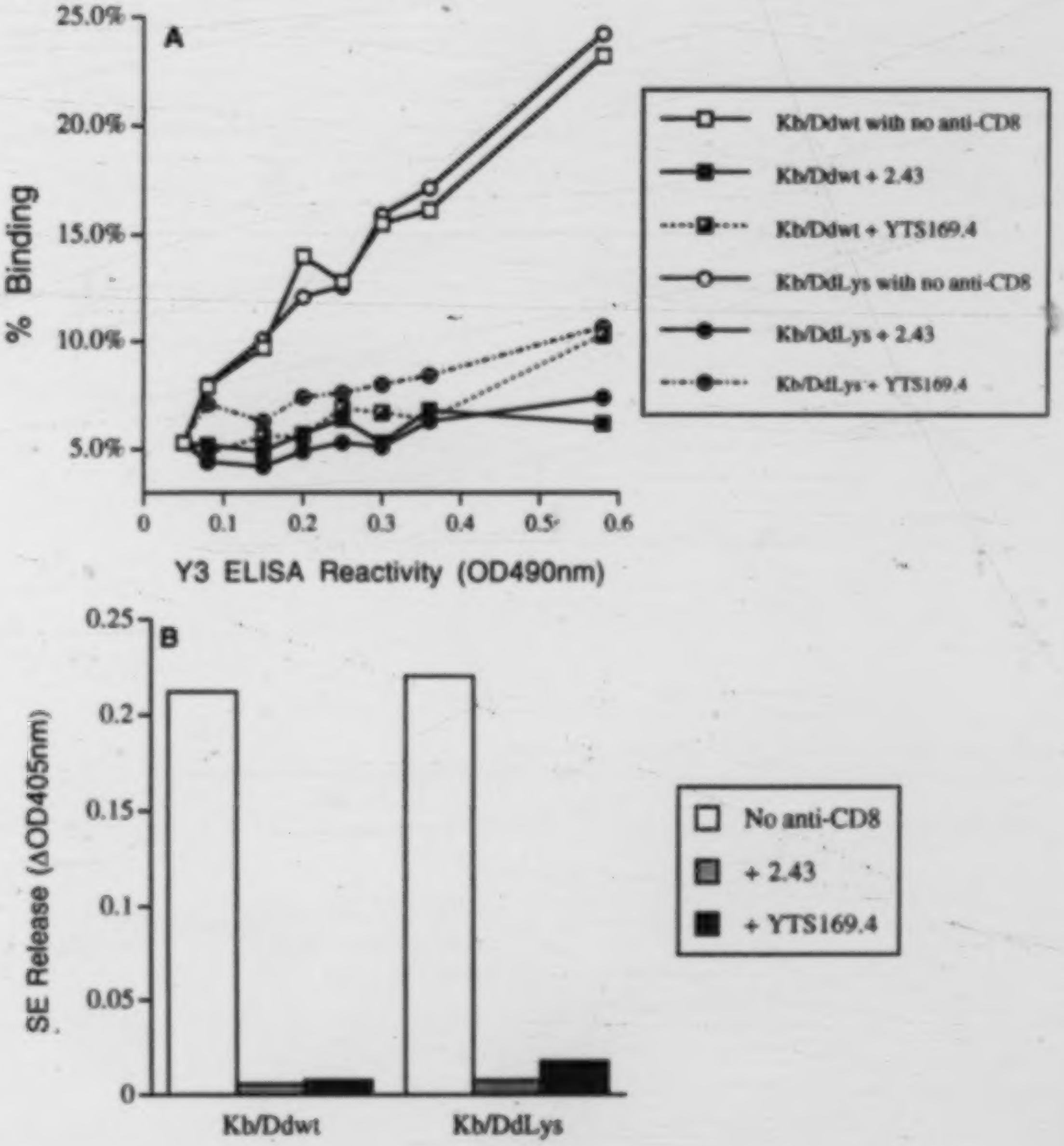


Figure 4-9. The pD^b/D^{d}wt and pD^b/D^{d}Lys $\alpha3$ domain mutant function equally well as CD8 accessory ligands for augmenting CTL binding and SE release when coimmobilized with suboptimal specific alloantigen. Purified K^b isolated from EL4 cells was immobilized on plastic at a suboptimal density of 0.02 μg/ml alone or together with the pD^b/D^{d}wt or pD^b/D^{d}Lys at the indicated densities. Clone 11 binding (A) and SE release (B) in response to coimmobilized suboptimal K^b and pD^b/D^{d}wt (*open squares*) or pD^b/D^{d}Lys (*open circles*) at various densities were determined. Binding of clone 11 to immobilized suboptimal K^b alone was 4.9%. Clone 11 SE release to suboptimal K^b Ag was at the background level (data not shown). Effects of the anti-CD8 mAb, 2.43, on suboptimal K^b Ag-triggered CTL binding (A) and enhanced degranulation response (B) to coimmobilized pD^b/D^{d}wt (*solid squares*) or pD^b/D^{d}Lys (*solid circles*) were also examined. Results are expressed as mean of triplicate wells.

Figure 4-10. The pK^b/D^{d}wt and pK^b/D^{d}Lys $\alpha3$ domain mutant function equally well as CD8 accessory ligands for augmenting CTL binding and SE release when coimmobilized with suboptimal D^b/NP complexes. The D^b/NP complexes were formed by immobilizing pD^b (isolated from EL4 cells) on plastic at a density of 0.021 µg/well and pulsing with 30 µM NP peptide. The pK^b/D^{d}wt or pK^b/D^{d}Lys molecules were coimmobilized at various densities. The standardization of pK^b/D^{d}wt or pK^b/D^{d}Lys was performed using solid phase ELISA with K^b-specific mAb Y3, and expressed as arbitrary OD values. In these experiments, OD 0.5 is equivalent to an input of 0.1 µg/well pK^b/D^{d}wt, and 0.13 µg/well pK^b/D^{d}Lys. (A) Clone 3/4 binding in response to coimmobilized suboptimal D^b/NP and pK^b/D^{d}wt (*open squares*) or pK^b/D^{d}Lys (*open circles*) at various densities was determined. Binding of clone 3/4 to immobilized suboptimal D^b/NP alone was 5.1%. (B) Clone 3/4 enhanced SE release in response to coimmobilized pK^b/D^{d}wt or pK^b/D^{d}Lys was determined. Both pK^b/D^{d}wt or pK^b/D^{d}Lys input were at an OD value of 0.5. Clone 3/4 SE release to suboptimal D^b/NP was at the background level (data not shown). Effects of the anti-CD8 mAbs, 2.43 and YTS169.4, on suboptimal D^b/NP-triggered CTL binding (A) and enhanced degranulation response (B) to coimmobilized pK^b/D^{d}wt or pK^b/D^{d}Lys were also examined. Results are expressed as mean of triplicate wells.

Figure 4-10


A
25.0%
20.0%
15.0%
10.0%
5.0%
% Binding
0
0.1
0.2
0.3
0.4
0.5
0.6
Y3 ELISA Reactivity (OD490nm)
Kb/Ddwt with no anti-CD8
Kb/Ddwt + 2.43
Kb/Ddwt + YTS169.4
Kb/DdLys with no anti-CD8
Kb/DdLys + 2.43
Kb/DdLys + YTS169.4
B
0.25
0.2
0.15
0.1
0.05
0
SE Release (ΔOD405nm)
Kb/Ddwt
Kb/DdLys
No anti-CD8
+ 2.43
+ YTS169.4

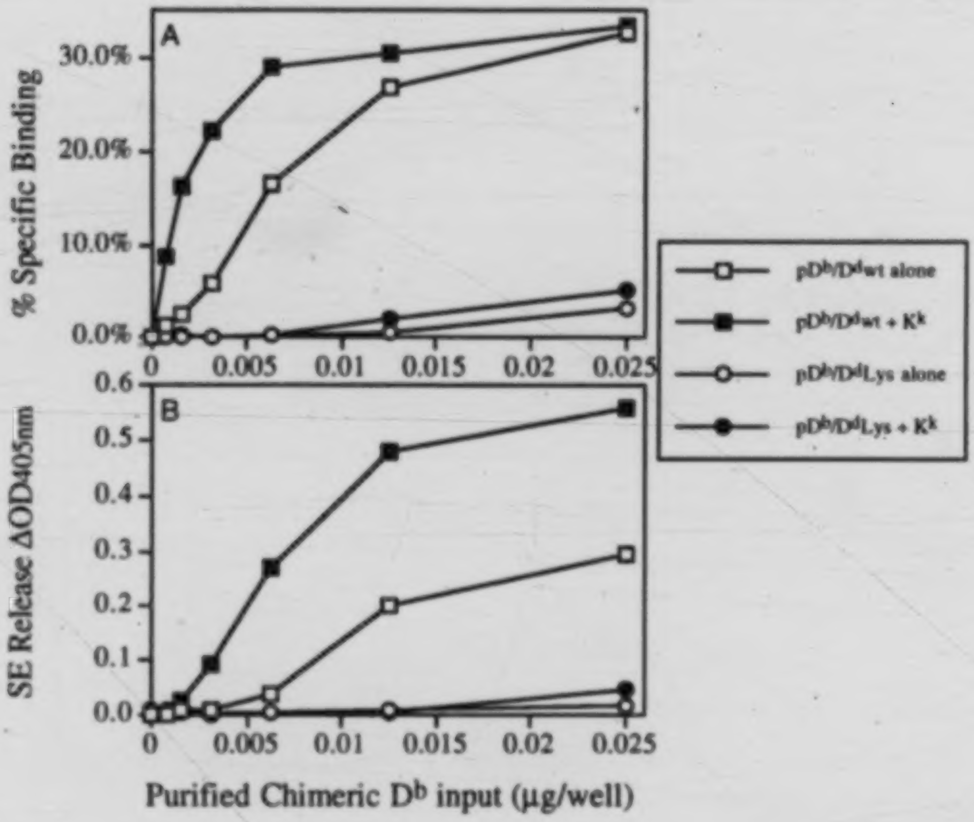


Figure 4-11. The pD^b/D^{d}Lys-NP peptide complexes are defective in initiating TCR-triggered avidity-enhanced CD8 adhesion to non-Ag class I and CTL response. Both the pD^b/D^{d}wt (*squares*) and pD^b/D^{d}Lys (*circles*) were titrated and immobilized separately on plastic with (*solid symbols*) or without (*open symbols*) the coimmobilization of a non-Ag class I molecules, K^k. Immunoaffinity purified K^k was used at a density of 0.20 µg/well. After pulsing with 30 µM NP peptide, 1.8 x 10^5 clone 3/4 CTL cells were loaded and incubated at 37°C for 4 h. Clone 3/4 binding (A) and SE release (B) were measured simultaneously. Non-specific binding to BSA was 5.2% and subtracted. Clone 3/4 binding to immobilized K^k alone was 2.3%.

CHAPTER V

GENERAL DISCUSSION

A. Roles of *N*-Linked Glycosylation in MHC Class I Biosynthesis.

Class I MHC heavy chains are glycoproteins. Murine MHC class I molecules have 2 or 3 *N*-linked glycans, while the human MHC class I has only one (Watts et al., 1989; Robinson and Kindt, 1989). Carbohydrate additions are important for glycoprotein biosynthesis, in that they protect from intracellular degradation, stabilize protein conformation and oligomerization, and regulate the egress of proteins to the cell surface. Consistent with this generalization, elimination of *N*-linked glycosylation sites by site-directed mutagenesis results in greatly reduced level of class I expression at the cell surface (Shiroishi et al., 1985; Miyasaki et al., 1986; Barbosa et al., 1987), suggesting an important role of glycosylation in MHC class I biosynthesis. Further studies have shown that the MHC class I molecule transiently associates with the chaperone protein calnexin in the ER, and this association is believed to augment the assembly of heavy chain/β2m complexes and facilitate peptide loading (Bergeron et al., 1994; Williams and Watts, 1995; Solheim et al., 1997). Interestingly, the initial interaction between class I and calnexin is dependent on carbohydrates on class I heavy chain (Zhang et al., 1995; Ware et al., 1995). Since the cell-surface Ly-m11$^+$ and M1/42^{high} K^b subsets appear to differ in their *N*-linked oligosaccharide(s) structures, they may undergo different post-translational modifications in the ER and the Golgi. It is currently unknown whether the potential differences in the ER glycosylation patterns of class I K^b subsets affects their ability to interact with calnexin and the peptide loading process. The observed *N*-linked glycosylation differences between the two K^b subsets are likely to be due to the modifications of the terminal oligosaccharide structures which occur in the post-ER apparatus. Since both K^b subsets are expressed on the cell surface and bear similar amounts of relevant endogenous peptides for allorecognition, it is unlikely that the potentially different glycosylation patterns of the intracellular forms of K^b subsets are dramatically affecting MHC class I biosynthesis and complex assembly. However, further studies are necessary to investigate the maturation kinetics and post-translational modifications of the two K^b subsets.

B. Roles of MHC Class I Glycosylation in TCR Recognition.

We have focused on the surface form of MHC class I molecules as TCR recognition structures. Crystal structures of both human and murine class I molecules have been resolved (Madden, 1995; Young et al., 1995). The MHC class I $\alpha 1$ and $\alpha 2$ domains form a peptide-binding groove with the α helices as sides and the β sheets as the bottom (Bjorkman et al., 1987; Fremont et al., 1992). This structural information is of particular interest with regard to the positions of class I *N*-linked glycosylation sites. The *N*-linked glycans at residue 86 and 176 are both in close proximity with the most variable region of class I heavy chain which forms part of the peptide-binding groove (Bjorkman et al., 1987; Parham, 1996a). Mutational analysis of MHC class I has shown that the TCR contacts a limited region of class I (Sun et al., 1995; Smith and Lutz, 1997) which immediately juxtaposes these glycosylation sites. Recent crystal structures of the TCR complexed with MHC/peptide have provided more detailed data (Garcia et al., 1996a; Garboczi et al., 1996). In these studies, the TCR is seen to contact the MHC class I molecule diagonally and, consistent with the mutagenesis data, the glycosylation sites are in close apposition to the TCR contact sites (Garcia et al., 1996a; Garboczi et al., 1996). Although the MHC class I oligosaccharide structures have been speculated to be pointing away from the TCR contact sites (Bjorkman et al., 1987), no information is available on their orientation. Furthermore, the oligosaccharide structures could adopt various conformations (Bush, 1992) and could be large in size (Wier and Edidin, 1988). Collectively, the location of the carbohydrates in close proximity to TCR contact sites in addition to the potential bulkiness and flexibility of these groups raise the intriguing possibility that they may interfere with TCR/MHC interactions (Shen and Kane, 1995; Parham, 1996b).

N-linked glycosylation of both human and mouse MHC class I has been reported not to be required for CTL recognition and effector functions (Goldstein and Mescher, 1985; Miyasaki et al., 1986; Barbosa et al., 1987), however, the complementary issue, i.e. the effects of over-glycosylation of MHC class I on CTL recognition, has not been directly entertained. We have succeeded in isolating two class I K^b subsets that differ significantly in their degrees of *N*-linked glycosylation, and more importantly, have made functional comparisons between these two class I species. Judging from their migration behaviors by SDS-PAGE and pI values by isoelectric focusing electrophoresis, the $M1/42^{high}$ K^b subset most likely represents the "normal" glycosylated form, while the Ly-m11$^+$ K^b subset apparently corresponds to the overglycosylated form. Both K^b subsets contain similar amounts of relevant peptides for allo-CTL recognition, however the overglycosylated form of K^b fails to effectively trigger CTL adhesion and responses. Given the large size and the complexity of the oligosaccharides on Ly-m11$^+$ K^b, it is tempting to speculate that the oversized carbohydrate structures sterically interfere with TCR interaction with K^b as appears possible based on the TCR/MHC crystal structural information. Such steric inhibition would prevent the CTL from recognizing the "masked" MHC class I molecule, even though it may contain the right peptide for recognition. The size or shape of oligosaccharide necessary to cause this type of "masking" effect is currently unknown. One of the carbohydrate modifications resulting from cell transformation is increased addition of poly-*N*-acetyllactosamine (PL) to *N*-linked carbohydrate chains (Saitoh et al., 1992). Our preliminary results suggest that PL is present on the Ly-m11$^+$ K^b subset and is partially responsible for the increased oligosaccharide content observed; it may also be responsible for the failure of this K^b subset to serve as a TCR ligand. Further efforts to characterize and define the structures of class I *N*-linked carbohydrates, especially those attached to the Ly-m11$^+$ K^b subsets, will provide new insight into the effects of glycosylation of MHC class I on TCR recognition.

C. MHC Class I as a NK Recognition Structure: Is There a Role for *N*-Linked Glycosylation?

In addition to serving as TCR ligand, MHC class I has been shown to function as a NK recognition structure that can provide a "negative" signal to turn the NK cell off (Reviewed in Gumperz and Parham, 1995; Lanier and Phillips, 1996; Colonna, 1996; Moretta, et al., 1996). In humans, the natural killer cell inhibitory receptors (KIR), which belong to the Ig-superfamily, specifically recognize polymorphic sites in the HLA class I $\alpha 1$ domain. In mouse, the ligands on NK cells for class I are the products of Ly-49 multigene family (Yokoyama and Seaman, 1993). It has been directly demonstrated that Ly-49A is a class I MHC binding receptor (Karlhofer et al., 1992; Kane, 1994; Brennan et al., 1994). Members of the Ly-49 family are type II integral membrane proteins homologous to the C-type lectin family suggesting that ligands for Ly-49 could be carbohydrate (Yokoyama and Seaman, 1993; Gumperz and Parham, 1995). Although the carbohydrate moieties of HLA class I do not seem to affect human KIR recognition (Gumperz et al., 1995), it has been shown that Ly-49A and Ly-49C recognize sulfated mono- and polysaccharides (Daniel et al., 1994; Brennan et al., 1995), demonstrating a potential role of class I oligosaccharide as a recognition structure of Ly-49 family members.

Although the carbohydrate on the Ly-m11$^+$ K^b subset might interfere with TCR recognition, it may be necessary for interaction with other class I binding receptors such as the Ly-49 family. It is possible that certain carbohydrate structures linked to K^b, perhaps as displayed by the Ly-m11$^+$ subset, may be required for recognition by certain Ly-49 family member(s). Since Ly-49C recognizes K^b and its recognition appears to require sulfated carbohydrate structures (Brennan et al., 1995), it will be interesting to determine if the Ly-m11$^+$ K^b contains sulfated carbohydrate structures and is a Ly-49C ligand.

D. Implications of MHC Class I Glycosylation on Immune Surveillance.

The two cell surface forms of K^b that differ in their abilities to trigger antigen specific CTL may reflect differences that occur in antigen presentation by class I *in vivo*. For example, different cells may express varying densities of the two functionally distinct K^b subsets, as suggested by the differing relative yields of the subsets from different cell lines, and this might, in certain circumstances, influence their abilities to serve as antigen presenting cells. From our results, peptides load into both subsets equally well, yet only the complexes of peptide and M1/42^{high} K^b serve as both a TCR and CD8 ligand and induce a response. If expression of the Ly-m11$^+$ K^b subset is high, it may compete with the M1/42^{high} subset for binding to a limiting peptide antigen, and thus maintain the density of the relevant ligand for TCR recognition at a level below that for an effective response. Further studies are necessary to determine whether this can indeed occur.

Our results also provide insights into the immune surveillance of tumor cells. Both CTL and NK cells play crucial roles in monitoring cancer transformations and eliminating tumors. However, the immune system generally fails to mount effective immune responses against invading tumors (Doherty et al., 1984; Ostrand-Rosenberg, 1994; Pardoll, 1995), because under immune pressure, *in vivo* grown tumor cells adopt various mechanisms to evade detection by the immune system. The loss and alteration of MHC class I expression has been proposed to be one of the factors responsible for the failure of the immune system to control tumor growth (Tanaka et al., 1988; Gopas et al., 1989; Garrido et al., 1995). Our results are consistent with this notion, and add one more complexity to this already very complicated issue. Tumor cells, especially those with a highly metastatic phenotype, are known to express altered carbohydrate moieties on some of their glycoproteins (Fukuda, 1985; Saitoh et al., 1992). It is conceivable that MHC class I could be one such structure that is expressed with altered carbohydrate structures on tumor cells. Altered glycosylation on MHC class I molecules could provide tumor cells with an effective means to escape both CTL and NK cell recognition. This is reminiscent of the strategies adopted by cytomegalovirus to evade immune surveillance, in which the virus interferes with the expression of MHC class I to escape CTL recognition, and coincidentally expresses an MHC class I homologue to turn NK cells off (Karre and Welsh, 1997). In the situation of tumors, the overglycosylation of MHC class I could diminish or prevent tumor cell recognition by the TCR, thus providing a means of escaping CD8$^+$ CTL surveillance; on the other hand, the altered oligosaccharide structures on MHC class I could enhance interaction with inhibitory NK receptors, i.e. Ly-49 or KIR, to downregulate NK activity leading to escape from NK cell killing. Tumor cells with downregulated MHC class I on the surface may effectively avoid CTL killing, but may be more susceptible to killing by NK cells. However, tumor cells with certain carbohydrate modifications could possibly escape both the CTL and NK cell destruction. Further *in vivo* experimentation with animal models are necessary to determine whether MHC class I glycosylations affects tumorigenicity and metastatic potential using tumor cell variants with various extents of oligosaccharide on MHC class I. These studies could potentially provide useful information for the design of better cancer vaccines.

E. Roles of MHC Class I Interaction with CD8 in CTL Recognition and Activation.

The above discussions have focused on the interaction of class I α1α2 polymorphic regions with TCR on the CTL. This interaction confers the specificity of T cell recognition and initiates the process T cell activation. It is important to appreciate that the class I α3 non-polymorphic domain interaction with CD8 on the surface of CTL is also critically involved in CD8-dependent CTL recognition and activation (Julius et al., 1993; Miceli and Parnes, 1993).

CTL activation involves the interaction of mutiple cell surface proteins with their respective ligands on target cells. It is the avidity level achieved by the mutiple receptor-ligand interactions (input) between the CTL and target that determines the outcome of CTL functionality (output), which is analogous to the avidity model for thymic selection of T cells. An avidity below a certain threshold (suboptimal) is not sufficient to induce CTL function, but does induce a state of unresponsiveness or anergy. On the contrary, if the avidity reached is too high (supraoptimal), CTL may undergo apoptotic death (Alexander-Miller et al., 1996). Only when the avidity is within an optimal range will CTL effector functions be carried out.

Parameters contributing to the overall avidity include TCR intrinsic affinity for MHC, number of participating TCR, number of MHC/peptide complexes, and involvement of CD8 and other accessory molecules (Alexander et al., 1991; Takeshita et al., 1993; Al-Ramadi et al., 1995; Kim et al., 1996; Schodin et al., 1996). Since the TCR usually has a low affinity for MHC, and the number of antigenic MHC/peptide complexes is low, CD8 is usually required to facilitate the interactions between TCR and MHC (Alexander et al., 1991; Takeshita et al., 1993; Cai and Sprent, 1996). Experimentally, if CTL effector functions can be readily blocked by anti-CD8 mAb, the CTL recognition and activation is considered CD8-dependent. As other variables are also involved in the CTL responses, CD8 dependency is relative. In our system, by varying the densities of immobilized MHC class I and antigenic peptides, we observed that there is relative resistance to CD8 mAb inhibition of CTL adhesion and response when the density of class I/peptide complex is high. This suggest that when TCR/MHC interactions are increased, the need for CD8 participation is therefore decreased.

Recently, an interesting but controversial model has been proposed about T cell activation. In this "serial engagement" or "serial triggering" model, each MHC molecule is re-used and serially triggers up to a few hundred TCR, and the T cell is activated when a threshold number of TCR have been triggered (downregulated or endocytosed) (Valitutti et al., 1995; Viola and Lanzavecchia, 1996). If this scenario is true, CD8 participation cannot simply be interpreted as facilitating T cell activation by enhancing avidity between CTL and targets, or between TCR and MHC class I. Based on this model, it would be "unfavorable" for TCR to have a high affinity and low off rate for MHC because this would presumbly hinder serial triggering. How then does CD8 fit in? It is possible that for class I to effectively trigger the TCR serially, a certain affinity of interaction must be maintained for a minimum time. CD8 could optimally enhance the affinity between the TCR and MHC class I, stabilize the complex, and prolong the interaction. Additionally, CD8 could function as a "positioning molecule" that facilitates a perfect fit between the TCR and MHC class I, or CD8 could induce a conformational change of the MHC/TCR complex favoring efficient TCR signaling (Garcia et al., 1996b). However, a recent report by Cai et al. (1997) suggests that TCR downregulation is not causally connected with the T cell activation process *per se*, and CD8 involvement in TCR downregulation was not demonstrated.

F. Dissection of CD8 Coreceptor and Accessory Molecule Functions.

The above discussion does not take into account the fact that CD8 serves dual functions during CTL recognition and activation. CD8 can function both as a "coreceptor" by binding to the same MHC class I molecules as the TCR, as well as an "accessory molecule" by binding to any class I molecules including those which do not interact with TCR (non-Ag class I) (O'Rourke et al., 1990; O'Rourke and Mescher, 1993; Kane and Mescher, 1993; Julius et al., 1993; Takeshita et al., 1993; Knall et al., 1994; Luescher et al., 1995). Both functions are important depending on the situation, but dissection of the two CD8 functions has been difficult. Earlier studies involving CD8 gene transfer into class I-restricted T cell hybrids (Dembic et al., 1987; Gabert et al., 1987) have clearly demonstrated a role for CD8 in enhancing T cell function, but it was not possible to clearly identify whether CD8 was serving as a coreceptor or an accessory molecule or both. Transfection of CD8 into MHC mismatched (class II-specific) CD4$^+$ T cell hybrids has demonstrated that CD8 can function as an accessory molecule (Ratnofsky et al., 1987; Miceli et al., 1991). In these MHC class II-restricted T cells, transfected CD8 could not bind class II molecules in a coreceptor manner, and yet it was found to enhance T cell hybrid IL-2 production. This enhancement of cytokine production was inhibitable by both anti-CD8 and anti-class I mAbs, and was dependent on the expression of MHC class I on the stimulators, suggesting that CD8 can interact with class I in an accessory fashion. However, whether CD8 underwent avidity enhancement and bound class I molecule was not directly demonstrated. Using the solid phase system, we can directly test whether CD8 functions as coreceptor or accessory molecule without the potential interference of other molecules on the cell surface.

Previous studies showed that an MHC class I $\alpha3$ domain mutant is defective in triggering effective CTL responses (Potter et al., 1990; Salter et al., 1990), however, it has been unclear whether the defect is that the mutant class I affect CD8 coreceptor function or CD8 accessory function or both. Luescher et al. (1995) showed that soluble wild type MHC class I molecules, but not class I with an $\alpha3$ domain mutation were able to bind TCR on CD8-expressing CTL, implying that the mutant class I molecules may prevent CD8 from functioning as a coreceptor. However no functional studies were undertaken. Taking advantage of our solid phase system, we have demonstrated and confirmed that the MHC class I $\alpha3$ domain acidic loop is a critical site for CD8 coreceptor binding, but is not essential for activated CD8 binding. Importantly, we are the first to provide functional data in support of this model.

The above discussion suggests that CD8 coreceptor and accessory functions can be dissected. It is possible that CD8-dependency can be subdivided based on these distinct CD8 functions as shown below:

TCR affinity x Number of TCR/MHC complexes

high ---> low

CD8-independent   CD8-dependent

1) Coreceptor-dep   2) Coreceptor-dep

Accessory-indep   Accessory-dep

(dep: dependent; indep: independent)

In CD8-independent CTL, Ag recognition and cellular activation are dependent on neither CD8 coreceptor nor accessory molecule function. In CD8-dependent CTL, dependency on CD8 coreceptor function is a defining prerequisite as we demonstrated that CD8 accessory function requires the antigen priming of TCR in which an effective CD8 coreceptor function is essential. In this scenario, the dependency on CD8 accessory function is determined by TCR/MHC affinity, the number of participating complexes, and involvement of other adhesion molecules. In a more physiological situation where the TCR affinity is low and only a limited numbers of MHC/peptide complexes are involved, CTL recognition and activation is usually dependent on both CD8 coreceptor and accessory functions (Kane et al., 1993; Takeshita et al., 1993; Corr et al., 1994).

Our results have demonstrated that the class I $\alpha 3$ mutant fails to initiate an efficient TCR signal transduction event due to its failure to serve as an effective CD8 coreceptor ligand. However, the biochemical basis for why class I $\alpha 3$ mutant fails to effectively trigger TCR is largely unknown. It has been shown that $\alpha 3$-mutated class I does not trigger CTL PI hydrolysis, a relative downstream signaling event (Knall et al., 1995), suggesting that CD8 coreceptor function is required for effective TCR signaling. A recent report by Madrenas et al. (1997) has shown that CD4 non-binding mutant class II molecules only induce partial T cell activation of the $CD4^+$ Th clones. In this system, phosphorylation of CD3 ε

chain and ZAP-70 was greatly reduced or undetectable, and phosphorylation pattern of ζ chain was altered (Madrenas et al., 1997); all these are characteristic of the signaling pattern of partial agonists/antagonists (Evavold et al., 1993; Sloan-Lancaster and Allen, 1996). It is tantalizing to speculate that CD8 coreceptor non-binding mutant class I molecules could behave in a similar fashion. Partial signaling through TCR due to defective CD8 coreceptor function is likely to be insufficient to activate CD8 and other adhesion molecules for their accessory function, and hence results in a greatly reduced T cell responses.

G. Mechanisms of TCR-Activated CD8 Binding to MHC Class I.

We have demonstrated that activated CD8 binding to MHC class I molecules can occur even when the class I molecules lack a critical CD8 binding site in the α3 domain. However, we can only speculate as to how the activated CD8 binds the α3-mutated class I (see Chapter IV, Discussion), mainly because the biochemical and biophysical mechanisms through which the TCR modulates the adhesiveness or the avidity of CD8 to MHC class I molecules are not well understood. Although the phenomenon of TCR-activated CD8 binding to MHC class I has been well documented, it is still largely a functional description. There are several potential mechanisms that could contribute to the observed activated CD8 binding to MHC class I.

Mechanisms that do not involve CD8 affinity changes.

It is possible that CTL activation induces the upregulation of CD8 expression on the surface of CTL, and this increased level of CD8 molecules bind MHC class I with higher total avidity without any changes to CD8 affinity. However, our preliminary results and the results of others (O'Rourke et al., 1990) argue against this hypothesis as CD8 upregulation was not detected upon a soluble anti-TCR or CD3 mAb stimulation which is effective in triggering avidity-enhanced CD8 binding. Another scenario is that CTL activation induces morphological changes, i.e. flattening or spreading, which increases the contact area between CTL and target. The increased surface contact may favor increased CD8-MHC class I interactions and the subsequent enhanced binding. In addition, TCR stimulation can trigger cytoskeletal reorganization that may result in CD8 aggregation at the contact region. It has been reported that about 20 to 50% of the surface CD8 molecules in human resting T cells are associated with the cytoskeleton, and the association can be modulated by T cell activation (Geppert and Lipsky, 1991). CD8 "clustering" could dramatically increase the density of CD8 at the contact site, potentially creating multivalent binding sites that greatly increase CD8 binding to MHC class I. The observation that TCR-triggered CD8 binding to MHC class I is cytoskeleton dependent (O'Rourke et al., 1991) is consistent with, but does not prove this notion. Currently, it is not known how CD8 associates with the cytoskeleton and how this association is modulated by TCR signals. Further investigation of these issues will provide insights into the mechanisms of activated CD8 binding.

Mechanism that involves CD8 affinity changes

We also entertain the possibility that activated CD8 binding to MHC class I involves TCR-induced CD8 affinity changes. In this model, TCR signals directly or indirectly modulate CD8 to undergo a conformational change ("inside-out" signaling), resulting in CD8 affinity upregulation, i.e. from a low affinity state to a high affinity state, similar to that observed for LFA-1. There is direct evidence that upon TCR stimulation, LFA-1 undergoes conformational changes as detected by mAb, and affinity upregulation as detected by direct affinity determination

(Canabas and Hogg, 1993; Lollo et al., 1993). Unfortunately, this type of information is currently lacking for CD8 after TCR stimulation. It is estimated by surface plasmon resonance assay that soluble (static state) CD8 molecules have a moderate affinity for immobilized MHC class I, independent of the MHC haplotype or its bound peptides. The K_D value for this interaction is between 10 to 30 μM (Garcia et al., 1996b). However, it is presently impossible to employ this system to measure the affinity between activated CD8 and MHC class I until more biochemical information is available for activated CD8. Although direct evidence demonstrating that CD8 undergoes affinity upregulation upon TCR stimulation is currently lacking, there are observations suggesting this is likely to occur: 1) TCR-initiated, PTK-mediated signaling events are essential for the expression of avidity-enhanced CD8 binding to class I (O'Rourke and Mescher, 1993). Upon CTL activation, CD8 α chain becomes serine/threonine phosphorylated (Acres et al., 1987; Blue et al., 1987; Williams et al., 1987). Although the significance of CD8 serine/threonine phosphorylation is unclear, it could provide a potential means of CD8 affinity modulation, perhaps through a phosphorylation-induced conformational change. 2) TCR stimulation has been reported to change the carbohydrate composition on CD8 β chains (Casabo et al., 1994). The carbohydrate structures on the CD8 β chain could potentially influence the physical structure and conformation of the CD8 heterodimers. 3) Certain anti-CD8 antibodies have been shown to have differential abilities to block activated CD8 binding to MHC class I relative to the low avidity CD8 coreceptor binding to MHC class I (Luescher et al., 1995). 4) Our results have demonstrated that activated CD8 binding to MHC class I does not require the intact acidic loop in class I, while this region is essential for the coreceptor function of CD8. The results described in 3) and 4) could be interpreted as suggesting that distinct CD8 binding sites are involved in the two different types of CD8-MHC class I interactions; it could also suggest that CD8 undergoes a conformational change, creates a different epitope and binds a different site on MHC class I. If so, this neoepitope may be preferentially recognized by some anti-CD8 mAbs, but not others. At present, however, no anti-CD8 mAb has been clearly demonstrated to be specific for activated CD8 molecules.

The above mentioned possibilities are not mutually exclusive. It is very likely that several mechanisms, i.e. CD8 aggregation or clustering and conformational changes, are operational simultaneously at the cell surface level. The elusiveness of the mechanism(s) of activated CD8 binding to MHC class I has become a major hurdle in our further understanding of this process and must be the focus of further research.

In contrast to TCR-activated, avidity-enhanced CD8 adhesion to immobilized non-Ag class I molecules observed for CD8$^+$ CTL, CD4$^+$ Th cells stimulated with anti-TCR mAb or antigenic peptide/class II complexes do not undergo an avidity-enhancement of CD4 to bind coimmobilized non-Ag class II molecules and augment T cell responses (O'Rourke and Lasam, 1995). This result suggests that the mechanism(s) of CD8 and CD4 coreceptor/accessory molecules in regulating T cell adhesion and responses are fundamentally different, although CD4 and CD8 are functionally analogous to each other. This could also be a reflection of the drastically different structures between CD4 and CD8 (Leahy, 1995)

H. CD8: Avidity Augmenter, Signaling Molecule, or Both?

Compelling evidence has clearly demonstrated that CD8 can enhance the avidity between CTL and target both as coreceptor and accessory molecule (O'Rourke et al., 1990; Garcia et al., 1996). In addition to its function as an avidity augmenter, CD8α has been reported to be associated with a *src*-related protein tyrosine kinase, $p56^{lck}$ (Veillette et al., 1988), and may therefore play a signaling role in CTL activation. Attempts to elucidate the functional significance of CD8-$p56^{lck}$ association in CTL maturation and activation have produced conflicting results. Two different systems have been employed. One is to transfect T cell hybridomas, in parallel, with intact CD8α or mutant CD8α that cannot associate with $p56^{lck}$, and look at the ability of these different CD8 forms to augment T cell responses. While most reports have demonstrated that CD8 association with $p56^{lck}$ is required for CD8 to augment T cell responses (Zamoyska et al., 1989; Letourneur et al., 1990; Miceli et al., 1991; Hoeveler and Malissen, 1993), others have shown that this association is not necessary (Miceli et al., 1991; Tanabe et al., 1992). It is difficult to reconcile these contradicting data, given that they have been obtained in similar systems. The observed differences could be due to the affinity of TCR, the basal activation states of the hybrids, and the participation of other adhesion molecules. It is of particular interest that when CD8 was assayed only for its ability to serve as an accessory molecule, the cytoplasmic tail of CD8 was not required to augment T cell IL-2 secretion (Miceli et al., 1991). In other cases where CD8 was required to serve as a coreceptor (and possibly as an accessory molecule simultaneously), a more stringent requirement for an intact cytoplasmic domain and/or $p56^{lck}$ association site was seen (Zamoyska et al., 1989; Letourneur et al., 1990; Hoeveler and Malissen, 1993). This could suggest that for effective coreceptor function of CD8, concomitant signaling capacity is required, while for accessory function of CD8, signaling through its cytoplasmic tail is a less stringent requirement. This makes teleological sense because the primary role of low avidity coreceptor CD8 is probably not to enhance the avidity between CTL and target, but to facilitate efficient TCR signaling for which CD8 cosignaling is important. Since only a small cellular fraction of CD8 is associated with $p56^{lck}$, it is possible that the subset of CD8 associated with $p56^{lck}$ preferentially serves as coreceptors, while those not associated with $p56^{lck}$ preferentially perform accessory functions via a distinct mechanism(s). Transfection of cytoplasmic-truncated or mutated CD8 into $CD8^-$ T cell hybrids and determination of CD8 accessory function in these transfectants could further clarify whether CD8 accessory function is dependent on CD8 signaling capacity.

Another approach used is to generate transgenic mice carrying intact CD8 or CD8 that is unable to associate with $p56^{lck}$ in CD8α gene-disrupted mice. In this system, an essential role of CD8α cytoplasmic tail or its association with $p56^{lck}$ for thymic selection has not been demonstrated (Levin et al., 1993; Chan et al., 1993; Fung-Leung et al., 1993; Smith et al., 1996), despite the fact that T cell development is greatly impaired in both CD8α gene-disrupted and $p56^{lck}$ gene-disrupted mice. It is noteworthy that in this system, 5 to 10 fold overexpression of $p56^{lck}$-non-associating CD8 was required to restore T cell development. These results suggest that CD8 lacking associated $p56^{lck}$ is less efficient in potentiating T cell development, although the CD8-$p56^{lck}$ association is not essential for this process. However, it is important to note that mature T cells and immature thymocytes may have significantly different signaling requirements for their activation. For instance, about 50% of the CD8 α chains expressed on thymocytes are not full length but the tailess form, α', which is unable to associate with $p56^{lck}$ (Zamoyska and Parnes, 1988; Zamoyska et al., 1989). This implies that for normal thymocyte development, the need for CD8-$p56^{lck}$ association is less stringent, and may explain why the CD8-$p56^{lck}$ association is not essential in the transgenic mice.

It should be noted that the above discussion does not exclude the possibility that signaling through accessory CD8 could still occur. In fact, O'Rourke and co-workers have found that soluble anti-TCR mAb activated CD8 binding to non-Ag class I can enhance T cell phosphoinositide hydrolysis and calcium flux (O'Rourke and Mescher, 1992 & 1994). The activated CD8 binding is inhibitable by protein tyrosine kinase and protein kinase C inhibitors, suggesting signaling events are involved. Along the same line, Anel et al. (1996) showed increased tyrosine phosphorylation of several proteins and decreased tyrosine phosphorylation of $p56^{lck}$ upon activated CD8 binding to class I. Interestingly, total cellular $p56^{lck}$ kinase activity increased after CD8 binding to MHC class I, however, whether CD8-associated $p56^{lck}$ activity also increased was not directly shown. We have consistently observed that soluble anti-TCR triggered CD8 binding to non-Ag class I induces significant tyrosine phosphorylation of an unidentified 42 kD protein in cloned CTL, suggesting that activated CD8 binding to MHC class I can generate biochemical signals which may participate in the CTL activation process.

In summary, it is likely that differential requirements exist for CD8 to serve different functions. For CD8 to serve its coreceptor function, its signaling (including that mediated by $p56^{lck}$) role is predominant. On the other hand, for CD8 to serve an accessory function, its primary role is to enhance the avidity between CTL and target, although some signaling does occur. Although many aspects of this hypothesis are still speculative, it provides a framework for future investigation.

I. Roles of CD8 α and β Chains in CD8 Coreceptor and Accessory Functions.

Early studies demonstrated that CD8 αα homodimers are sufficient for T cell maturation, binding to MHC class I molecules and CD8-dependent CTL functions, however the role of CD8 β chain has often been ignored despite the fact that CD8 αβ heterodimer is the more physiological form involved in CTL function (Zamoyska, 1994). Accumulating evidence suggests that CD8 β chains are involved in T cell maturation as well as mature T cell function. Transfection of CD8 β genes into CD8 α-expressing T cell hybridomas can further enhance IL-2 production (Wheeler et al., 1992; Karaki et al., 1992). CD8 β gene knock-out mice display reduced number of mature CD8$^+$ T cells as well as an impaired negative selection process (Nakayama et al., 1994; Fung-Leung et al., 1994; Crooks and Littman, 1994). Although the CD8 β chain itself does not seem to be involved in signaling, it can assist the α chain in recruiting p56^{lck} for signaling (Irie et al., 1995). These observations clearly demonstrate a role of CD8 β chain in T cell development and activation.

The relative contributions of CD8 α and β to CD8 coreceptor and accessory functions are less clear. As demonstrated by photoaffinity labeling and anti-CD8 antibody blocking, CD8 αβ heterodimers appear to be more efficient at facilitating soluble MHC class I binding to specific TCR than CD8 αα homodimers (Luescher et al., 1995; Renard et al., 1996), suggesting that the CD8 β chain can play a role in CD8 coreceptor function. However, the mechanism through which the CD8 β chain contributes to CD8 accessory function remains undetermined. Although CD8 αα homodimers are sufficient to mediate static-state CD8 adhesion to MHC class I (Salter et al., 1990; Garcia et al., 1996b), it is unknown whether the CD8 β chain is required for TCR-triggered, activated CD8 binding to MHC class I, since the previous experiments demonstrating activated CD8 binding were performed using CTL expressing CD8 αβ heterodimers (O'Rourke et al., 1990; Kane and Mescher, 1993; Luescher et al., 1995). As the basal binding of CD8 αα homodimer-expressing B3Z cells to MHC class I is not detectably increased, this could suggest that CD8 β chain does not serve to prevent low avidity CD8 α binding. Since CD8 αα homodimers are sufficient to mediate TCR-triggered, avidity-enhanced binding to MHC class I, any role(s) of CD8 β chain in modulating this type of CD8-class I interaction is likely to be subtle. Alternatively, the primary function of the CD8 β chain may be to enhance CD8 low-affinity coreceptor function(s) (Zamoyska, 1994; Garcia et al., 1996b; Shen et al., 1996; Renard et al., 1996 a & b).

J. A Two-Step Model of CD8 Involvement during CTL Activation.

Since CD8 can serve both coreceptor and accessory functions during T cell activation, a question arises as to whether the CD8 coreceptor and accessory functions are segregated or interdependent. Previous reports suggest that effective CD8 accessory function is dependent on and subsequent to TCR stimulation (O'Rourke et al., 1990; Kane and Mescher, 1993). However, these results do not provide information on the role of CD8 coreceptor function in the expression of avidity-enhanced CD8. By using an α3 domain mutated MHC class I molecule which is unable to serve effective CD8 coreceptor function, we have demonstrated that the activated CD8 accessory function is dependent on productive CD8 coreceptor function through which CD8 facilitates efficient TCR signaling. Based on these results, a two-step model for the roles of CD8-MHC class I interaction during CD8-dependent CTL activation is proposed. As an initial step, specific TCR interacts with MHC class I-peptide complex, but this interaction is not sufficient to initiate signaling events to activate other adhesion processes. At this stage, low-avidity CD8 comes in to interact with MHC class I at the α3 domain and stabilizes the TCR/MHC interaction for the initial signaling events, and serves a coreceptor function. This TCR/MHC/CD8 trimolecular complex should be considered as a "signaling unit" and is essential for the subsequent activation events of CD8-dependent CTL. Once this trimolecular complex is formed, it transduces signals, including activation of PTK p56^{lck} and ZAP-70, necessary for the second step avidity upregulation of CD8 and likely other adhesion molecules to achieve increased avidity between the CTL and target for a full T cell activation. The avidity-enhanced CD8 serves an accessory function by binding all MHC class I, including those not recognized by TCR, in a manner that is independent of a critical CD8 binding site in the acidic loop of class I $\alpha3$ domain. This model highlights the differential functions of CD8 as coreceptor as well as accessory molecule and clarifies the manner through which CD8 molecules function in concert to orchestrate efficient CTL activation.

CHAPTER VI

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